

8-2014

TYROSINE 370 PHOSPHORYLATION OF ATM POSITIVELY REGULATES DNA DAMAGE RESPONSE

Hong-Jen Lee

Follow this and additional works at: http://digitalcommons.library.tmc.edu/utgsbs_dissertations

 Part of the [Cancer Biology Commons](#)

Recommended Citation

Lee, Hong-Jen, "TYROSINE 370 PHOSPHORYLATION OF ATM POSITIVELY REGULATES DNA DAMAGE RESPONSE" (2014). *UT GSBS Dissertations and Theses (Open Access)*. Paper 487.

This Dissertation (PhD) is brought to you for free and open access by the Graduate School of Biomedical Sciences at DigitalCommons@The Texas Medical Center. It has been accepted for inclusion in UT GSBS Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@The Texas Medical Center. For more information, please contact laurel.sanders@library.tmc.edu.

**TYROSINE 370 PHOSPHORYLATION OF ATM POSITIVELY REGULATES
DNA DAMAGE RESPONSE**

by

HONG-JEN LEE, M.S.

APPROVED:

Supervisory Professor: Mien-Chie Hung Ph.D.

Dihua Yu, M.D., Ph.D.

Pierre D. McCrea, Ph.D.

Bin Wang, Ph.D.

Shiaw-Yih Lin, Ph.D.

APPROVED:

**Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston**

**TYROSINE 370 PHOSPHORYLATION OF ATM POSITIVELY REGULATES
DNA DAMAGE RESPONSE**

A

DISSERTATION

**Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment**

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

HONG-JEN LEE, M.S.

Houston, Texas

Aug, 2014

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my mentor Dr. Mien-Chie Hung, who always shares with me his philosophy, scientific thinking, attractive ideas and constructive suggestions and supports me whenever I need his help. I really enjoyed and appreciated for each moment when we discussed about science together. I would like also thank all my committee members, Drs. Dihua Yu, Shiaw-Yih Lin, Pierre D. McCrea, Bin Wang, Elsa R.Flores, Michael W. Van Dyke and Mong-Hong Lee for their generosity and kindness to share with me their experience and helpful advices.

I thank my colleagues and collaborators including Drs. Li Lan, Guang Peng, Wei-Chao Chang, Ming-Chuan Hsu, Ying-Nai Wang, Chien-Chia Cheng, Leizhen Wei, Satoshi Nakajima, Chung-Hsuan Chen Shih-Shin Chang, and Hsin-Wei Liao, for their helps and contributions to my research project. I am thankful to my friends Jung-Mao Hsu and Hsu-Ping Kuo for sharing with me a nice working environment Lab S.

I do appreciate for all the supports, encouragements, patience and love from my wife, my families and friends in Taiwan.

TYROSINE 370 PHOSPHORYLATION OF ATM POSITIVELY REGULATES DNA DAMAGE RESPONSE

Hong-Jen Lee, M.S.

Advisory Professor: Mien-Chie Hung, Ph.D.

Abstract

Ataxia telangiectasia-mutated (ATM) mediates DNA damage response by controlling irradiation (IR)-induced foci formation, cell cycle checkpoint, and apoptosis. However, how upstream signaling regulates ATM is not completely understood. Here, we show that upon IR stimulation, ATM associates with and is phosphorylated by epidermal growth factor receptor (EGFR) at Y370 at the site of double-strand breaks. Depletion of endogenous EGFR impairs ATM-mediated foci formation, homologous recombination, and DNA repair. Moreover, ATM Y370F mutant or pretreatment with an EGFR kinase inhibitor gefitinib blocks EGFR and ATM association, hinders CHK2 activation and subsequent foci formation, and increases radio-sensitivity. Thus, we reveal a critical mechanism by which EGFR directly regulates ATM activation in DNA damage response and ATM Y370 phosphorylation may serve as a biomarker for radiation and anti-EGFR combinational therapies.

TABLE OF CONTENTS

DEGREE OF APPROVAL SHEET	i
TITLE OF DISSERTATION	ii
ACKNOWLEDGMENTS.....	iii
ABSTRACT	iv
TABLE OF CONTENTS	v
TABLE OF ILLUSTRATIONS	x
CHAPTER 1 INTRODUCTION	1
1.1 DNA damage response	1
1.2 Biological functions and significance of ATM.....	3
1.3 Post translational modifications of ATM.....	6
1.4 EGFR signaling pathway and cancer	8
1.5 Biological functions of nuclear EGFR	10
1.6 The role of nuclear EGFR in DNA damage response.....	12
1.7 Combinational therapy of EGFR inhibitors and radiation.....	14
1.8 Hypothesis.....	15
CHAPTER 2 MATERIALS AND METHODS.....	18

2.1 Nuclear fractionation, Western blotting and immunoprecipitation assays	18
2.2 Chromatin enriched fractionation.....	19
2.3 Immunofluorescence staining for irradiation-induced foci formation....	20
2.4 KillerRed system	21
2.5 Laser microirradiation	22
2.6 Neutral comet assay.....	23
2.7 Cell culture, transient transfection, and viral infection	23
2.8 <i>In vitro</i> kinase assay	24
2.9 Chromatin immunoprecipitation assay.....	25
2.10 Mass spectrometry analysis	25
2.11 Homologous Recombination (HR) repair analysis.....	26
2.12 Colony formation assay	26
CHAPTER 3 ATM IS PHOSPHORYLATED AT TYROSINE 370 (Y370)	
UPON IR STIMULATION	27
3.1 Identification of ATM phosphorylation sites by Mass spectrometry analysis	27
3.2 ATM Y370 is evolutionary conserved from yeast to mammals	28
CHAPTER 4 EGFR MEDIATES ATM TYROSINE PHOSPHORYLATION.....	31

4.1 Identification of kinase mediating ATM tyrosine phosphorylation	31
4.2 ATM is phosphorylated by EGFR <i>in vitro</i> and <i>in vivo</i>	31
CHAPTER 5 EGFR ASSOCIATES WITH ATM IN THE NUCLEUS IN RESPONSE TO IR	37
5.1 Endogenous EGFR associates with ATM in the nucleus upon IR stimulation.	37
5.2 EGFR C-terminal CR domain is required for ATM interaction.	38
CHAPTER 6 EGFR IS RECRUITED TO DSBs IN RESPONSE TO DNA DAMAGE STIMULI	43
6.1 EGFR co-localizes with ATM at <i>I-SceI</i> induced DSBs.	43
6.2 EGFR is recruited to DSBs in response to laser light activation.	43
CHAPTER 7 EGFR CO-LOCALIZES WITH ATM AND γ-H2AX AT DSBs	50
7.1 EGFR co-localizes with ATM and γ -H2AX at laser irradiation- induced DSBs.	50
7.2 EGFR associates with MRN complex in the nucleus.....	50
CHAPTER 8 EGFR IS REQUIRED FOR ATM AND CHK2 IRIF	55
8.1 EGFR is required for ATM IRIF	55
8.2 Depletion of EGFR impairs CHK2 IRIF.....	55
CHAPTER 9 EGFR IS REQUIRED FOR ATM-MEDIATED DNA REPAIR	61

9.1 Knocking down-EGFR reduces DNA repair ability.	61
9.2 EGFR is required for ATM-mediated homologous recombination repair.	62
CHAPTER 10 EGFR MEDIATES ATM Y370 PHOSPHORYLATION.....	66
10.1 ATM Y370 is a major EGFR-mediated phosphorylation site.	66
10.2 Generation of AYM pY370 antibody.	66
CHAPTER 11 EGFR MEDIATES DDR via ATM Y370 PHOSPHORYLATION	72
11.1 ATM Y370 phosphorylation facilitates ATM IRIF.	72
11.2 ATM Y370 phosphorylation defective mutant impairs downstream CHK2 IRIF and NBS1 S343 phosphorylation.	72
11.3 EGFR regulates DNA-PKcs activation through ATM Y370 phosphorylation.	73
CHAPTER 12 EGFR MEDIATES RADIO-THERAPY RESISTANCE THROUGH ATM Y370 PHOSPHORYLATION	79
12.1 ATM Y370 phosphorylation is essential for ATM-mediated radio- therapy resistance.	79
12.2 ATM-mediated radio-therapy resistance depends on EGFR kinase activity.	79

CHAPTER 13 SUMMARY AND DISCUSSION	84
13.1 Summary	84
13.2 The role of EGFR in cancer cell radio-sensitivity.....	87
13.3 Tyrosine kinases mediate ATM signaling.	88
13.4 The connections among ATM, DNA-PKcs and EGFR.....	88
CHAPTER 14 FUTURE DIRECTIONS.....	90
14.1 Hit and stay model.....	90
14.2 ATM conformational changes in response to DSBs formation.	91
14.3 Combination therapy of targeting nuclear EGFR and radiation	92
BIBLIOGRAPHY	94
VITA	113

TABLE OF ILLUSTRATIONS

Figure 1 The protein kinase structure and post-translational modifications of ATM.	5
Figure 2 ATM activation and downstream signaling pathways.	7
Figure 3 Model depicting project hypothesis.....	17
Figure 4 ATM is phosphorylated at tyrosine 370 (Y370) upon IR stimulation.	29
Figure 5 EGFR mediates ATM tyrosine phosphorylation.....	33
Figure 6 Endogenous EGFR associates with ATM in the nucleus upon IR stimulation.....	39
Figure 7 EGFR is recruited to DSBs in response to DNA damage stimuli....	45
Figure 8 EGFR co-localizes with ATM and other DDR proteins at DSBs.	51
Figure 9 EGFR is required for ATM and CHK2 IRIF.....	57
Figure 10 EGFR involves in DNA repair.	63
Figure 11 ATM Y370 phosphorylation is mediated by EGFR.....	68
Figure 12 EGFR mediated ATM Y370 phosphorylation facilitates ATM IRIF and downstream signaling	74
Figure 13 ATM Y370 phosphorylation regulates radio-sensitivity.	81
Figure 14 Propose model showing the role of EGFR in ATM-mediated DNA damage response.....	86

CHAPTER 1 INTRODUCTION

1.1 DNA damage response

DNA damage response (DDR) consists of massive signaling network and maintains genomic stability when mammalian cells are subjected to exogenous damage agents such as: radiation, radiomimetic chemicals, cancer therapeutic agents, or endogenous stimuli like reactive oxygen species and replication errors (Jackson and Bartek, 2009). Among various DNA lesions, DNA double strand breaks (DSBs) formation is one of the most injurious lesions to DNA structure and chromatin integrity (Bensimon et al., 2011). Proteins involve in DDR can be classified into three categories: sensors, transducers and effectors (Zhou and Elledge, 2000). Sensor proteins in DDR, such as: Ku70-Ku80 heterodimer, Mre11-Rad50-NBS1 (MRN) complex or γ -H2AX, are those proteins recognize broken DNA fragments in a sequence-independent manner and being recruited to DNA breaks at early time points after DNA damaged (McGowan and Russell, 2004). Then those sensor proteins recruits more signal transducers, including three major serine/threonine phosphoinositide-3 kinase like kinases (PIKK): ataxia telangiectasia mutated (ATM), ATM Rad3-related protein (ATR) and the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), to the DSBs through protein-protein interactions (Lempiainen and Halazonetis, 2009). Such

protein recruitments and accumulations at DSBs form foci and further facilitate signal transduction by post translational modification (PTM). Next, transducer proteins initiate series of signaling cascades to downstream effectors for execution of cell cycle arrest, DNA repair, foci formation, transcription or apoptosis.

Although ATM, ATR and DNA-PKcs all serve as important transducers in DDR, they are activated by various DNA damage stimuli and involved in different signaling pathway (Ditch and Paull, 2012). ATR is activated by single stranded DNA lesions formation or collapsed replication forks, through binding with ATR-interacting protein (ATRIP), and recruited to the region coated by replication protein A (RPA) (Cimprich and Cortez, 2008; Flynn and Zou, 2011; Lopez-Contreras and Fernandez-Capetillo, 2010; Shiotani and Zou, 2009). ATM and DNA-PKcs are both activated by DSBs induction but associating with diverse protein complex and initiating different DNA repair signaling pathways. ATM is recruited to DSBs by association with MRN complex and activated via autophosphorylation at Serine 1981 (S1981) and regulates homologous recombination (HR) DNA repair (Derheimer and Kastan, 2010; Lavin and Kozlov, 2007; Lee and Paull, 2007).

In contract, DNA-PKcs engages in non-homologous end-joining (NHEJ) DNA repair through association with Ku70-Ku80 heterodimers at

DSBs (Dobbs et al., 2010; Mahaney et al., 2009; Nagasawa et al., 2011). However, how mammalian cells command the signaling directions between HR (ATM/MRN complex) or NHEJ (DNA-PKcs/Ku heterodimers) remains unclear. Accumulating evidence showed that failure of DNA repair or impeding appropriate DDR may result into cell death, chromosomal deletion, translocation or mutation and then contribute to promote tumor initiation.

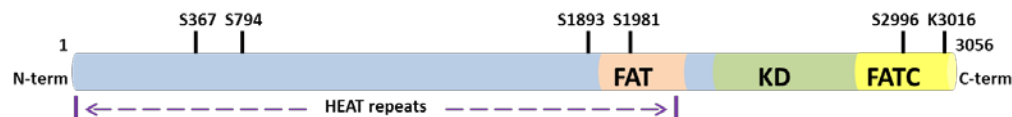
1.2 Biological functions and significance of ATM

Ataxia telangiectasia (A-T) is a rare, inherited neurodegenerative disease which results into difficulties of movement and coordination, impairment of immune system and predisposition of cancer (Zhang et al., 1997). Due to missense or nonsense mutation of *ATM* gene, most A-T patients do not possess functional ATM protein kinase which plays a critical role in maintaining genomic stability. *ATM* gene encodes a 350 kDa protein kinase which consists of N-terminal HEAT (huntingtin, elongation factor 1A, protein phosphatase 2A A-subunit, TOR) repeats (Perry and Kleckner, 2003), FAT domain (FRAP, ATM and TRRAP; aa 1960–2566), PI-3 kinase like kinase domain (KD domain, aa 2712–2962) and C-terminal FATC (FRAP, ATM and TRRAP C-terminal; aa 2963–3056) domain (Figure 1).

With treatments of radiation or agents induce DSBs formation, inactive ATM homodimer dissociates as active monomer and autophosphorylates at Serine 367, Serine 1893, Serine 1981 and Serine 2996 (Bakkenist and Kastan, 2003; Bensimon et al., 2010; Kozlov et al., 2011; Kozlov et al., 2006) (Figure 2). Previous studies demonstrated that mutations of those ATM autophosphorylation sites disrupt ATM-mediated DDR signaling pathways in human cells. However, mouse model of ATM mutations and *in vitro* studies showed that those ATM autophosphorylation sites are dispensable for ATM activation by DNA damage stimuli or oxidation (Daniel et al., 2008; Guo et al., 2010; Lee and Paull, 2005; Pellegrini et al., 2006), suggesting ATM activation may require additional regulation mechanisms.

Figure 1 The protein kinase structure and post-translational modifications of ATM.

Schematic representation of ATM protein kinase structure, positions of autophosphorylation serine residues (S367, S1893, S1981 and S2996), CDK5-mediated phosphorylation residue (S794) and TIP60-mediated acetylation residue (K3016). N-term: N-terminal. C-term: C-terminal. HEAT repeats: huntingtin, elongation factor 1A, protein phosphatase 2A A-subunit, TOR. FAT domain: FRAP, ATM and TRRAP; aa 1960–2566. KD domain: PI-3 kinase like kinase domain, aa 2712–2962. FATC domain FRAP, ATM and TRRAP C-terminal; aa 2963–3056.



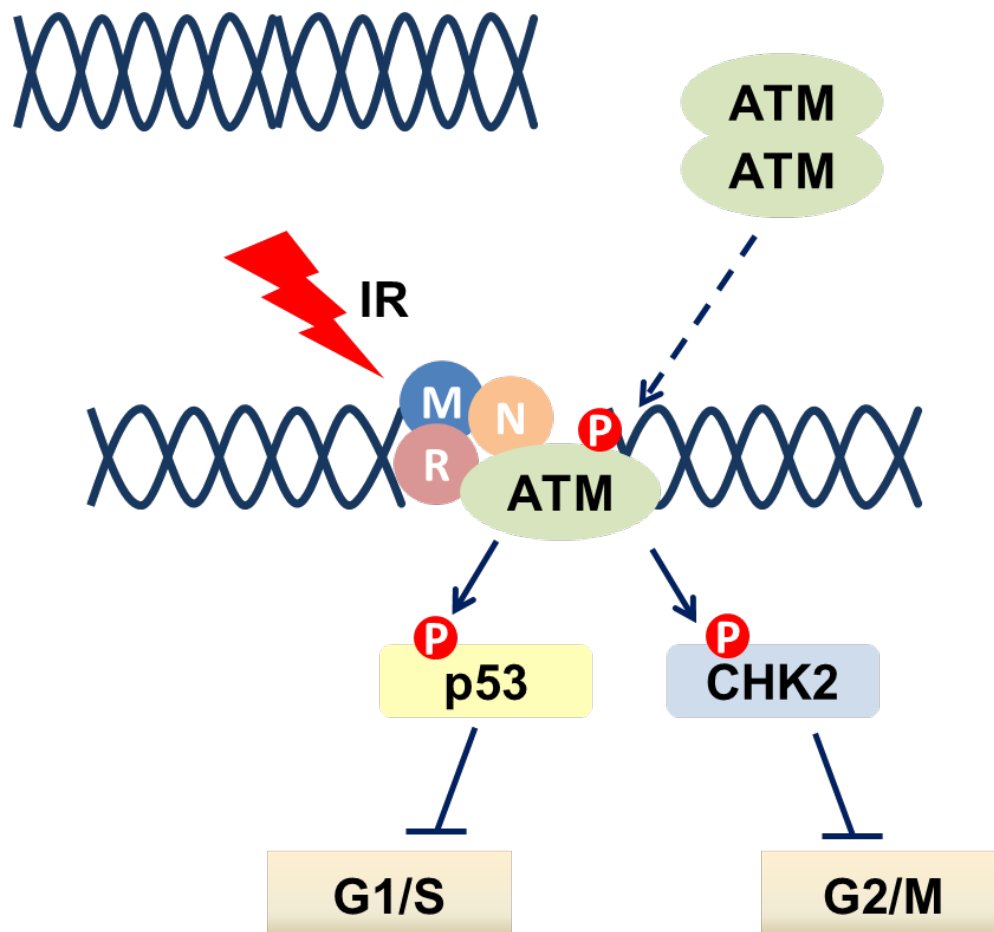
As a serine/threonine kinase, ATM transduces signals in DDR network through mediating more than seven hundred putative substrates phosphorylation (Matsuoka et al., 2007), which is identified in a global proteomic analysis. When recruited to DSBs and associated with MRN complex, ATM subsequently phosphorylates checkpoint kinase 2 (CHK2) at Serine 68 or p53 at Serine 15 to regulate cell cycle checkpoint or apoptosis (Barlow et al., 1997; Matsuoka et al., 2000) (Figure 2). In addition to serving as a component of MRN complex which accounts for ATM DSBs recruitment, Nijmegen breakage syndrome 1 (NBS1) is also an ATM substrate. It had been shown that ATM-mediated NBS1 serine 278 and serine 343 phosphorylation facilitates structural maintenance of chromosome 1 (SMC1) phosphorylation and cell cycle arrest in S phase (Kitagawa et al., 2004; Yazdi et al., 2002).

1.3 Post translational modifications of ATM

Several important post translational modifications (PTM) which affect ATM functions and activity had been identified by different research groups. In post-mitotic neurons, cyclin-dependent kinase 5 (CDK5) directly phosphorylates ATM at serine 794 (S794) upon DNA damage stimulation (Tian et al., 2009). Mutation of S794 impairs ATM S1981 autophosphorylation and downstream substrates p53 and H2AX functions.

Figure 2 ATM activation and downstream signaling pathways.

Upon ionizing radiation (IR) stimulation, inactive homodimer ATM dissociates as active monomers and is recruited to DSBs by MRN complex. Subsequently, activated ATM transduces signal to downstream substrates like p53 or CHK2 through protein-protein interaction and phosphorylation and mediates cell cycle arrest for DNA repair. M: Mrell. R: Rad50. N: NBS1. P: phosphorylation.



Moreover, blockage of CDK5-ATM signaling pathway protects neuron cell death induced by DNA damage agents, implying that CDK5 mediating ATM S794 phosphorylation plays a critical role in ATM activation. By using a systematic mutagenesis assay, ATM lysine 3016 (K3016) acetylation, mediated by Tip60 histone acetyltransferase, was shown to be required for ATM activation and downstream p53 or CHK2 phosphorylation (Sun et al., 2007). Mutation of ATM K3016 not only abolishes ATM kinase upregulation but also hinders dissociation of ATM inactive homodimer to active monomer, suggesting ATM K3016 acetylation may play a key role in ATM activation.

1.4 EGFR signaling pathway and cancer

The epidermal growth factor receptor (EGFR) is a well-documented oncogene and plasma membrane abundant receptor tyrosine kinase (RTK) which functions at the upstream of various signaling cascades. It belongs to ErbB (erythroblastic leukemia viral oncogene homolog) family including EGFR (ErbB-1), HER2 (ErbB-2), ErbB-3 and ErbB-4 (Yarden and Sliwkowski, 2001). Ligands, such as epidermal growth factor (EGF), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AREG), transforming growth factor- α (TGF- α), betacellulin (BTC), epigen (EPG) or epiregulin (EPR), stimuli trigger EGFR homodimerization or

heterodimerization with other ErbB proteins (Ferguson et al., 2003; Garrett et al., 2002; Ogiso et al., 2002; Olayioye et al., 2000; Yarden and Sliwkowski, 2001). Such EGFR conformational changes lead to kinase activity upregulation and subsequently autophosphorylation at multiple tyrosine (Y) residues including Y992, Y1045, Y1068, Y1086, Y1148 and Y1173 of the C-terminal region (Linggi and Carpenter, 2006; Olayioye et al., 2000; Zhang et al., 2006).

EGFR gene mutation or ligands amplification, resulting into EGFR signaling upsurge which leads to tumor initiation, progression or metastasis, was observed in types of cancer like breast cancer, head and neck cancer, lung cancer, colorectal cancer and glioblastoma (Holbro et al., 2003; Normanno et al., 2005; Normanno et al., 2006; Uberall et al., 2008; Zandi et al., 2007). Once activated, EGFR rapidly associates with Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain containing proteins such as growth factor receptor-bound protein 2 (GRB2) and recruits a cascade of protein kinase to regulate cell cycle, cell survival and proliferation (Yaffe, 2002). The recruitments and interactions between EGFR and adaptor proteins like GRB2 initiate several signaling networks including: the PI3K-AKT pathway (PI3K, phosphoinositide 3-kinase), the RAS-RAF-MEK-ERK pathway (Ras, rat sarcoma viral oncogene homologue; RAF, v-raf murine sarcoma viral

oncogene homologue; MEK, MAPK/ERK activator kinase; ERK, extracellular signal-regulated kinase), the STATs (signal transducer and activator of transcription) pathway and the PLC γ -PKC pathway (PLC γ , phospholipase C- γ PKC, protein kinase C) which serve critical roles in regulation of cancer cell adhesion, migration, invasion, proliferation and transformation (Bogdan and Klambt, 2001; Jorissen et al., 2003; Yarden and Sliwkowski, 2001; Zandi et al., 2007). Monoclonal antibodies against EGFR (targeting extracellular domain) and small molecular chemical compounds targeting EGFR kinase domain had been developed and used in clinical for cancer patient treatments or combinational therapies (Tebbutt et al., 2013).

1.5 Biological functions of nuclear EGFR

Other than the role of EGFR in traditional signaling pathways, increasing evidence demonstrated by different group of researchers showed that some populations of EGFR localizing in the nucleus involve in gene transcription, cell proliferation, DNA repair, and chemotherapy or radiotherapy resistance in the last decade (Wang and Hung, 2009; Wang et al., 2010). Mechanisms triggering plasma membrane bound EGFR translocate from cell surface to the nucleus can be classified as two types: ligand-dependent and ligand-independent. In response to EGF ligand

stimulation, nuclear EGFR associates to the A-T rich sequence (ATRS) residing in *cyclin D1* promoter, turns on downstream genes expression and increases cancer cell proliferation (Lin et al., 2001). Although EGFR does not possess DNA binding domain, one group demonstrated that nuclear EGFR can target another ATRS in *iNOS* (inducible nitric oxide synthase) promoter region through binding with transcriptional factor STAT3 (Lo et al., 2005). Similar to this study, nuclear EGFR was shown to transactivate *Aurora-A* promoter by cooperating with STAT5 and amplify *Aurora-A* gene expression, which leads to chromosomal instability and tumorigenesis (Hung et al., 2008).

B-MYB is another transcriptional target of nuclear EGFR upon EGF stimulation (Hanada et al., 2006). In breast cancer cell, nuclear EGFR and E2F1 regulate B-MYB gene expression through targeting *B-MYB* promoter in an EGFR kinase activity dependent manner. As a tyrosine kinase, nuclear EGFR was shown to physically associate with PCNA (proliferating cell nuclear antigen) and phosphorylate PCNA at tyrosine 211 (Y211) residue (Wang et al., 2006). They also showed that EGFR-mediated PCNA Y211 phosphorylation is not only required for chromatin-bound PCNA stability but also correlates with nuclear EGFR expression level, suggesting PCNA Y211 phosphorylation may serve as a marker for overall survival rate of breast cancer patient.

When discussing the role of nuclear EGFR, one important issue needs to be addressed is how membrane-bound EGFR translocates from plasma cell surface to the nucleus. To this point, accumulated data showed that early stage of EGFR internalization and the association between EGFR and Importin β 1 are critical for EGFR nuclear entry through nuclear pore complex (Lo et al., 2006). However, the interaction between EGFR and exportin CRM1 mediates EGFR nuclear export. Besides, a putative nuclear localization sequence (NLS), containing three clusters of basic amino acids, identified in EGFR intracellular carboxyl terminus participates into EGFR nuclear localization as well (Hsu and Hung, 2007). In fact, EGFR is not the only RTK can be found in the nucleus among all the ErbB family proteins. It had been shown that full-length ErbB2 and ErbB3 can both be detected in the nucleus by using immunohistochemical (IHC) staining, immunofluorescence staining and transmission electron microscopy (TEM). (Offterdinger et al., 2002; Wang et al., 2004; Xie and Hung, 1994).

1.6 The role of nuclear EGFR in DNA damage response

In ligand-independent pathway, EGFR can be directed to the nucleus in response to radiation, UV, H₂O₂ or therapeutic chemical compounds stimuli (Wang and Hung, 2009; Wang et al., 2010). DNA-

PKcs, one of the upstream signal transducer in DDR and regulating NHEJ repair pathway when DSBs formation, is the most well-documented protein involving in nuclear EGFR functions in DDR. In 1998, nuclear EGFR was first reported to associate with DNA-PKcs and Ku70-Ku80 heterodimers in the nucleus by using immunofluorescence staining and confocal microscopy (Bandyopadhyay et al., 1998). Pretreatment of c225 (cetuximab), a monoclonal antibody against EGFR extracellular domain not only reduces EGFR kinase activity but impairs DNA-PKcs nuclear localization and activity, implying the link between nuclear EGFR and DNA-PKcs.

Similar phenomenon was observed by different group who studied the role of nuclear EGFR in DDR. They found that radiation induces EGFR nuclear translocation, association with DNA-PKcs and consequently increases DNA-PKcs activity and radiosensitivity of cancer cells (Dittmann et al., 2005a; Dittmann et al., 2005b). Blockade of EGFR nuclear translocation by monoclonal antibody cetuximab, EGFR and HER-2 dual kinase activity inhibitor lapatinib or cyclooxygenase-2 (COX-2) inhibitor celecoxib impairs DNA-PKcs kinase activity and function in DNA repair and sensitizes cancer cells to radiation treatment (Dittmann et al., 2005b; Dittmann et al., 2008b; Kim et al., 2009). In non-small-cell lung cancer cells (NSCLC), mutant EGFR carrying with common oncogenic

tyrosine kinase domain mutations, L858R or DeltaE746-E750 elevates NSCLC radiosensitivity due to defect of nuclear translocation upon radiation treatment, suggesting the significance of nuclear EGFR in radiation therapy (Das et al., 2007).

1.7 Combinational therapy of EGFR inhibitors and radiation

Since EGFR orchestrates upstream signaling pathways in various types of cancer and radiation activating EGFR had been linked to radiotherapy resistance, pre-clinical tests which combine EGFR inhibitors with radiation therapy had become attractive and been conducted. In 2005, researchers observed that combination of EGFR kinase inhibitor erlotinib (Tarceva) with radiation can increase cell radiosensitivity and enhance cell apoptosis. Moreover, systemic administration of erlotinib profoundly shrinks tumor size when combining with radiation treatment in xenograft mouse model (Chinnaiyan et al., 2005).

By using monoclonal antibody cetuximab combining with radiation, similar result reported by another group indicated such a combination therapy demonstrates a cooperative growth inhibitory effect in certain NSCLC cell lines and xenograft mouse model (Raben et al., 2005). Another study, which combined with clinically using EGFR kinase inhibitor gefitinib and radiation, carried out in NSCLC cell lines showed that

gefitinib delays DSBs repair efficiency and increases cell radiosensitivity comparing with radiation treatment alone (Tanaka et al., 2008).

As a matter of fact, EGFR inhibitors sensitizing cancer cell in response to radiation treatment was also observed in head and neck squamous-cell carcinoma (HNSCC) cell lines (Harari and Huang, 2001; Huang et al., 1999; Huang and Harari, 2000; Loeffler-Ragg et al., 2008; Milas et al., 2000). To apply those pre-clinical discoveries to clinical site, a multinational, randomized clinical study performed with more than four hundred advanced squamous-cell carcinoma of the head and neck patients indicated that cetuximab combines with radiotherapy improved patient survival rate (Bonner et al., 2006).

1.8 Hypothesis

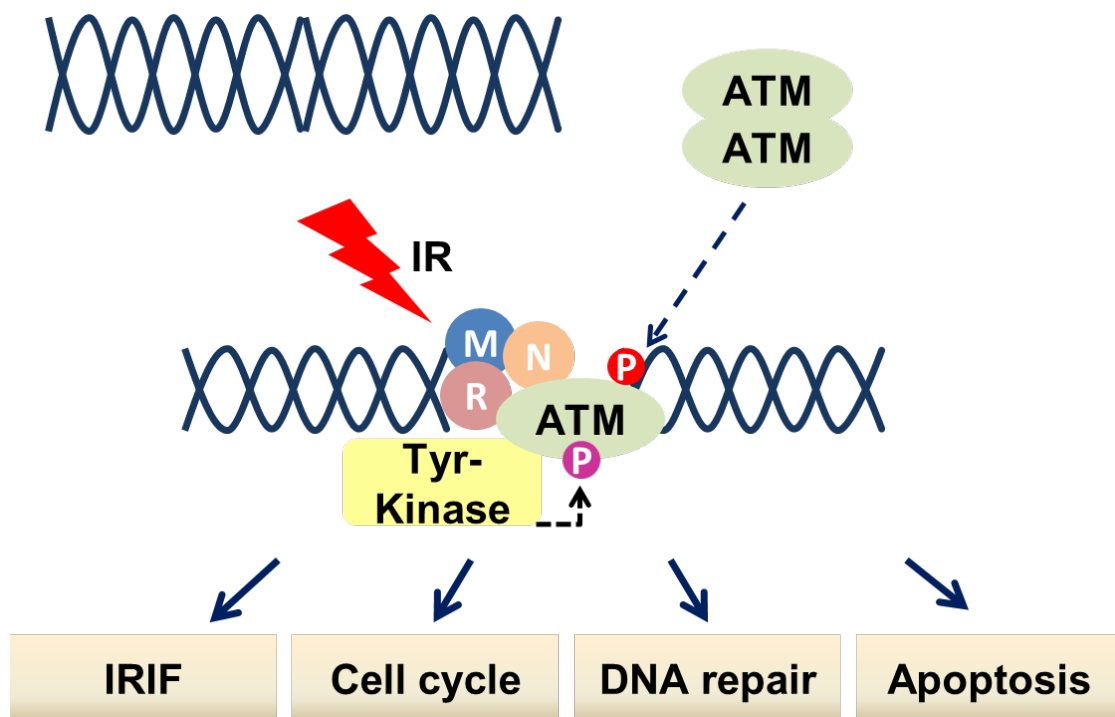
Several lines of evidence have demonstrated that autophosphorylation of ATM at S367, S1893, and S1981 are individually required for ATM activation and ATM-mediated DDR in human. However, more recent studies have indicated that mutation of either the prominent S1987 autophosphorylation site (corresponds to S1981 in human) or the three conserved autophosphorylation sites S367/S1899/S1987 (correspond to S367/S1893/S1981 in human) of ATM in mice had no effect on ATM-dependent response (Daniel et al., 2008; Pellegrini et al.,

2006). These findings suggest that other mechanisms may be involved in DNA damage-induced activation of ATM in addition to S367/S1893/S1981 autophosphorylation. We performed a mass spectrometry analysis and identified additional IR-triggered ATM phosphorylation at S85, Y370, T1885, S1891, and S2592 (Figure 4A).

Since ATM is a serine/threonine kinase, the results suggest that ATM Y370 phosphorylation would require a tyrosine kinase and this tyrosine kinase may involve in mediation of DDR through regulating this ATM Y370 phosphorylation (Figure 3).

Figure 3 Model depicting project hypothesis

Since our preliminary results showed ATM is tyrosine phosphorylated at Y370 in response to IR stimulation, we propose that tyrosine kinase (Tyr-kinase) mediating this Y370 phosphorylation event may regulate ATM functions in DDR. P in red circle: ATM autophosphorylation S1981. P in purple circle: ATM Y370 phosphorylation.



CHAPTER 2 MATERIALS AND METHODS

2.1 Nuclear fractionation, Western blotting and immunoprecipitation assays

Nuclear fractionation and Western blotting were performed as described previously (Yu et al., 2012). Briefly, after serum starvation overnight, cells were treated with or without IR, washed with phosphate buffer saline (PBS) twice, and resuspended in lysis buffer (20 mM HEPES pH7.0, 10 mM KCl, 2 mM MgCl₂, 0.5% Nonidet P-40, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, and 2 µg/ml aprotinin) on ice. After homogenizing cells with 25-30 strokes in Dounce homogenizer and centrifugation at 1,500 x *g* for 5 min, supernatant was separated as cytosolic extract (CE) and nuclear pellets were wash with lysis buffer for three times and lysed in TGN buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Tween 20, 0.3% NP-40, 1 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, and 1X protease inhibitor mixture; Roche) (Yang and Kastan, 2000) with sonication.

Both nuclear extracts (NE) and CE were collected from supernatant after centrifugation at highest speed for 10 min at 4°C and protein concentration was determined by using BCA protein assay kit (Thermo Scientific). At least 2 mg of NE was applied to subsequent immunoprecipitation (IP) assay. Anti-EGFR (SC-03, 1:1,000; Santa

Cruz), anti-ATM (1:1,000; Bethyl Lab), anti-Lamin A (1:2,000; Santa Cruz), anti-tubulin (1:10,000; Sigma), anti-Flag (1:2,000; Sigma), anti-Myc (1:10,000; Roche), anti-phospho-EGFR Y1086 (1:2,000; Cell Signaling) and anti-phosphotyrosine (4G10, 1:3,000; Millipore) antibodies were used in western blotting. Antibodies against phospho-NBS1 S343 (1:2,000; Cell Signaling), NBS1 (1:40,000; Novus), Rad50 (1:10,000; GeneTex), Mrell (1:20,000; GeneTex), phospho-ATM S1981 (1:4,000; R&D system) and ORC2 (1:2,000; Cell Signaling) were used in detection of DNA damage response signaling. For immunoprecipitation, 5 µg of anti-phosphotyrosine antibody (4G10; Millipore), 2 µg of anti-EGFR antibody (Ab-13, Thermo Scientific) or 5 µg of anti-ATM antibody (Bethyl Lab) was used in 2 mg of nuclear extracts, respectively.

2.2 Chromatin enriched fractionation

EGFR knockdown (shRNA EGFR #1 and #2) or vector control HeLa cells were treated with or without 10 Gy IR. After IR, cells were washed by ice-cold PBS twice, resuspended with buffer A (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, and 1X protease inhibitor cocktail mixture (Roche)) and gently rotated at 4°C for 30 min. After

centrifugation at 6,500 x *g* for 5 min, pellets were washed with 1mL buffer A (without Triton X-100) once, resuspend with 1 mL of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and 1X protease inhibitor mixture), and gently rotated at 4°C for 30 min. After centrifugation at 6,500 x *g* for 5 min, pellets were washed with 1 mL of wash buffer I (3 mM EDTA, 0.2 mM EGTA, 10 mM Tris-HCl, pH8.0, and 150 mM NaCl) and gently rotated at 4°C for 15 min. This step was repeated with wash buffer II (3 mM EDTA, 0.2 mM EGTA, 10 mM Tris-HCl, pH8.0, and 250 mM NaCl). Cell pellets were lysed with TGN buffer, sonicated, and applied to centrifugation at 16,000 x *g* for 10 min at 4°C. Chromatin-enriched fraction was collected from the supernatant for subsequent Western blot analysis.

2.3 Immunofluorescence staining for irradiation-induced foci formation

For detection of p-ATM S1981 irradiation-induced foci formation (IRIF), EGFR-depleted HeLa cells were exposed to 2 Gy or 10 Gy IR and subjected to staining as previously described¹³. To detect whether ATM Y370F impaired p-ATMS1981 or p-Chk2 T68 IRIF, ATM-depleted HeLa cells were transfected with pcDNA3 vector alone, Flag-tagged ATM wild type (WT) or Y370F by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Post transfection for 24 h, cell transfectants were seeded on cover slides for subsequent IRIF staining as previous procedure. For detection of p-CHK2 IRIF, U2OS or HeLa cells were washed with PBS three times, fixed and permeabilized as previously described (Peng et al., 2009) then subjected to staining. Antibodies against phospho-ATM S1981 (1:200; Rockland Immunochemicals), phospho-CHK2 T68 (1:200; Cell Signaling), Flag (1:200; Cell Signaling), and phospho-H2AX S139 (1:200; Cell Signaling) were used in detection of IRIF.

2.4 KillerRed system

KillerRed (KR; Figure 7D) is a light-stimulated ROS-inducer fused to a tet-repressor (tetR-KR), which binds to a TRE cassette (~ 90 kb) integrated at a defined genomic locus in U2OS cells (U2OS TRE cell line) (Lan et al., 2010). KR facilitates the formation of oxygen radicals and superoxide through the excited chromophore (Carpentier et al., 2009; Pletnev et al., 2009) to induce DNA damage. By targeting the expression of KR to one specific genome site, we can visualize the recruitment of proteins at genetic loci. To activate KR, tetR-KR was exposed to 559 nm laser light for 50 scans (over a total of 10 s) at a power rate of 1 mW/scan (equal to 50 mW).

At “the KR-TRE array” induced localized damage, we have detected γ -H2AX at the site of tetR-KR but not tetR-monomer cherry (tetR-mcherry) after laser light exposure (Lan et al., 2013). For bleaching KR, a 559 nm laser (1 mW/scan) in a selected area was used (FV1000 SIM Scanner set with 405 nm laser diode, Cat. F10OSIM405, Olympus). The dose that was delivered to the KillerRed spot was calculated based on the pixel size, the pixel size for irradiation is (0.138 μ m/pixel) and the dwell time per pixel is (8 μ s/pixel). The irradiation is at 1.0 mW (1.0 mJ/s). With a dwell time of 8 μ s/pixel, this irradiates each pixel with 8.0 nJ/pixel/scan. Multiplying by the number of scans gives the total energy per pixel.

2.5 Laser microirradiation

The Olympus FV1000 confocal microscopy system was employed (Cat. F10PRDMYR-1, Olympus, UPCI facility) and FV1000 software was used for acquisition of images. For inducing DNA damage, cells are irradiated with 405 nm laser irradiation. The output power of the laser (original 50mW) passed through the lens was 5 mW/scan. Laser light was passed through a PLAPON 60x oil lens (super chromatic aplan. corr. obj W/1.4NA FV, Cat. FM1-U2B990). Cells were incubated at 37°C on a thermo-plate (MATS-U52RA26 for

IX81/71/51/70/50; metal insert, HQ control, Cat. OTH-I0126) in Opti-MEM during observation to avoid pH change.

2.6 Neutral comet assay

Neutral comet assay was carried out to determine the levels of DSB in EGFR-depleted and control HeLa cells according to the manufacturer's instruction of Trevigen's Comet Assay Kit (4250-050-K, R&D systems). The comet tail movement was measured by using CometScore software (www.autocomet.com) among more than 100 randomly selected cells in each experiment.

2.7 Cell culture, transient transfection, and viral infection

HeLa cell, U2OS cell, and HEK 293T cell were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum. All cell lines are characterized as mycoplasma negative. For transient transfection, cells were transfected with indicated plasmids by either SN liposome (Zou et al., 2002) or Lipofectamine 2000 (Invitrogen). For lentiviral short hairpin RNA (shRNA) infection, HEK 293T cells were cotransfected with vector control, two various EGFR-targeting or two different ATM-targeting shRNA with packing plasmids including

deltaVPR8.2 and envelope plasmid VSVG using SN liposome or Lipofectamine 2000 according to the manufacturer's instructions. Virus-particles containing media was harvested to infect HeLa cell or U2OS cell after 48 h cotransfection. All the infected cells were maintained in media containing 0.5 µg/mL puromycin.

2.8 *In vitro* kinase assay

Equal amounts of purified GST, GST-tagged ATM2 or ATM2/Y370F protein were incubated with purified recombinant human EGFR kinase (#7706, Cell Signaling), 10 µCi [γ -³²P] ATP, 10 µM ATP in kinase assay buffer (5 mM MgCl₂, 5 mM MnCl₂, 50 µM Na₃VO₄, 50 mM HEPES, pH7.4, and 5 mM DTT) at 30°C for 30 min. The kinase reaction was stopped by addition of SDS samples buffer and boiling. The samples were analyzed by SDS-PAGE, transferred to PVDF membrane, and detected by autoradiography. To detect EGFR directly phosphorylated full-length Flag-tagged ATM, purified Flag-tagged ATM protein was incubated with or without purified recombinant human EGFR kinase in the kinase assay buffer as previously mentioned without [γ -³²P] ATP. The resulting signal was detected by Western blotting with the indicated antibody.

2.9 Chromatin immunoprecipitation assay

To detect EGFR or ATM localized at DSB sites, DR-GFP integrated U2OS cells were transiently transfected with vector alone or I-SceI plasmid as described previously (Peng et al., 2009). After transfection for 24 h, transfectants were harvested for nuclear fractionation and intact nuclear pellets were collected for crosslink with formaldehyde and subsequent ChIP assays by using EZ-ChIP kit (Upstate) in accordance with the manufacturer's instructions. Anti-EGFR (Thermo Scientific, Ab-13) and anti-pATM S1981 (Rockland Immunochemicals) antibodies were used for immunoprecipitation. PCR was carried out with primers flanking the I-SceI restriction enzyme cutting site shown in Figure 7A.

2.10 Mass spectrometry analysis

Exogenous overexpressed Flag-tagged ATM was isolated from HeLa cell nuclear extracts by immunoprecipitation using anti-Flag antibody and analyzed by SDS-PAGE. To identify novel phosphorylation sites on ATM, mass spectrometry analysis was carried out as previously described (Liu et al., 2012).

2.11 Homologous Recombination (HR) repair analysis

To generate EGFR knockdown stable clones, U2OS cells containing a single copy of the HR repair reporter substrate DR-GFP were infected by lentiviral shRNAs targeting to EGFR or vector control, respectively. After 48-h transfection with mock or I-SceI plasmids followed by 16-h sodium butyrate (5 mM) treatment, flow cytometry analysis was carried out to determine the number of HR-repaired GFP-positive cells.

2.12 Colony formation assay

HeLa cells were re-plated and exposed to various doses of ionizing radiation (IR) the next day. After 10-day culture, cells were fixed with Acetic acid:methanol (1:3) solution and colonies were stained with 0.5% Crystal violet. The number of colonies was quantitated and results were normalized to plating efficiency.

CHAPTER 3 ATM IS PHOSPHORYLATED AT TYROSINE 370 (Y370) UPON IR STIMULATION

3.1 Identification of ATM phosphorylation sites by Mass spectrometry analysis

Since recent studies have shown that mutation of either the prominent S1987 autophosphorylation site (corresponds to S1981, which phosphorylation level serves as ATM activation marker in human cells) or the three conserved autophosphorylation sites S367/S1899/S1987 (correspond to S367/S1893/S1981 in human) of ATM in mice had no effect on ATM-dependent downstream signaling (Daniel et al., 2008; Pellegrini et al., 2006). These observations suggest that other mechanisms may be involved in DNA damage-induced activation of ATM in addition to S367/S1893/S1981 autophosphorylation.

To elucidate unknown ATM phosphorylation sites that may regulate ATM functions in DDR, we performed a mass spectrometry analysis and identified additional IR-triggered ATM phosphorylation at S85, Y370, T1885, S1891, and S2592 (Figure 4A). Among these, S85, T1885 and S1891 have been reported by other groups (Bennetzen et al., 2010; Kozlov et al., 2011; Matsuoka et al., 2007), which further substantiates the reliability of this mass

spectrometry analysis. However, detailed functions of ATM S85, T1885 and T1891 in DDR are not clear.

3.2 ATM Y370 is evolutionary conserved from yeast to mammals

Next, we aimed to figure out whether any ATM phosphorylation residue we identified in mass spectrometry analysis is evolutionary conserved. Between the two novel phosphorylation sites Y370 and S2592 identified, Y370 (Figure 4B) was shown to be evolutionary conserved from yeast to mammals (but not frog or fruit fly) (Figure 4C), suggesting this Y370 phosphorylation may involve in mediating functions in DDR. Since ATM is a serine/threonine kinase, the results suggest that ATM Y370 phosphorylation would require a tyrosine kinase.

Figure 4 ATM is phosphorylated at tyrosine 370 (Y370) upon IR stimulation.

A. Mass spectrometry analysis results of immunoprecipitated (IP) exogenous Flag-tagged ATM from HeLa cell nuclear extracts after 10 Gy IR. Sequence: ATM peptide analyzed by mass spectrometry. Position: phosphorylation residue on ATM.

sequence	position	modification
IAKPNVSA <u>S</u> TQASR	Ser85	Phospho
SLEISQSY <u>Y</u> TTTQR	Tyr370	Phospho
ST <u>T</u> PANLD <u>S</u> ESEHFFR	Thr1885, Ser1891	Phospho
SLAFEEG <u>S</u> QSTTISSLSEK	Ser1981	Phospho
QS <u>S</u> QLDEDRTEAANR	Ser2592	Phospho

B. Mass spectrometry image shows ATM is phosphorylated at Y370 (label as red) after 10Gy IR stimulation.

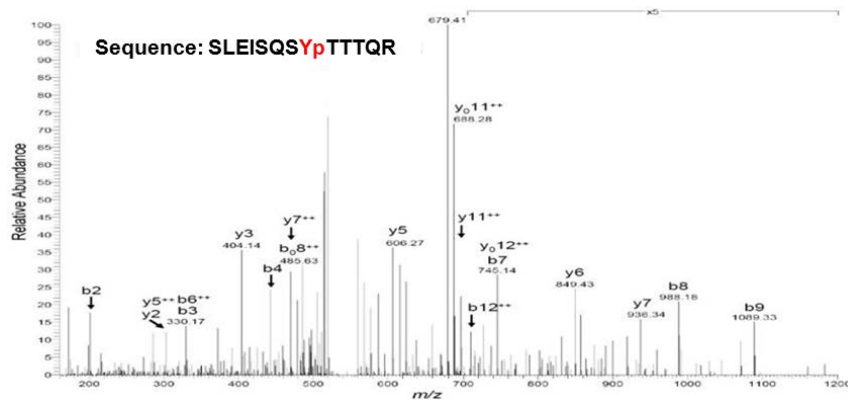


Figure 4 ATM is phosphorylated at tyrosine 370 (Y370) upon IR stimulation.

C. Alignment of ATM partial sequences from human to yeast. Red Y indicates human ATM tyrosine 370 (Y370) is conserved among all mammalian species and yeast. Frog possesses shorter form of ATM, which lacks the N-terminal region.

	Ser85 ↓	Tyr370 ↓
human (<i>Homo sapiens</i>)	-----SASTQA-SRQKKM	SLEISQ---SYTTTQRES
chimpanzee (<i>Pan troglodytes</i>)	-----SASTQA-SRQKKM	SLEISQ---SYTTTQRES
horse (<i>Equus caballus</i>)	-----SASTQA-SRQKKM	SLEISQ---SYATTQREC
dog (<i>Canis familiaris</i>)	-----SASTQA-TRQKKM	SLEISQ---SYTTTQREF
rabbit (<i>Oryctolagus cuniculus</i>)	-----SASTQA-SRQKKM	SLEISQ---SYTTTQRES
mouse (<i>Mus musculus</i>)	-----SATTQS-SRQKKM	SVEISQ---SYV-TQRES
frog (<i>Xenopus tropicalis</i>)	-----	-----
fruitfly (<i>Drosophila melanogaster</i>)	TQFNLEAGSQT-SGNGHF	-----KADEN
yeast (<i>Saccharomyces cerevisiae</i>)	-----STTNKLSLSENRL	-LGITKSLTYFALNRKN
	Thr1885 Ser1891 ↓ ↓	Ser1981 ↓
human (<i>Homo sapiens</i>)	TSRSTTPANLDSESEHFF	SLAFEEGSQNTTISSLSE
chimpanzee (<i>Pan troglodytes</i>)	TSRSTTPANLDSESEHFF	SLAFEEGSQSTTISSLSE
horse (<i>Equus caballus</i>)	TSRSTTPANLDSESEHIF	SLTFEEGSQSTTISSLSE
dog (<i>Canis familiaris</i>)	TSRSTTPANLDSESEHFF	SLTFEEASQNTAISSLSE
rabbit (<i>Oryctolagus cuniculus</i>)	TSRSTTPANLDSDSEHFF	SLTFEEGSQNTTISSLSE
mouse (<i>Mus musculus</i>)	ASRSATPANSDESSENFL	SPTFEEGSQGTTISSLSE
frog (<i>Xenopus tropicalis</i>)	-----	-----
fruitfly (<i>Drosophila melanogaster</i>)	----TAP-----NSQEIF	-----
yeast (<i>Saccharomyces cerevisiae</i>)	-----	YLLFEEMNMPN-I-----
	Ser2592 ↓	
human (<i>Homo sapiens</i>)	ITKNVPKQSSQLDEDRTE	
chimpanzee (<i>Pan troglodytes</i>)	ITKNVPKQSSQLDEDRTE	
horse (<i>Equus caballus</i>)	ITKSAPKQSSQLDEDRTE	
dog (<i>Canis familiaris</i>)	ITKNAPKQSSQLDEDRTE	
rabbit (<i>Oryctolagus cuniculus</i>)	ITKNAPKQSSQLDEDRME	
mouse (<i>Mus musculus</i>)	ITKSTSKENSHLDEDRTE	
frog (<i>Xenopus tropicalis</i>)	LTKNAPKQISQLDEDRME	
fruitfly (<i>Drosophila melanogaster</i>)	-----NTERSG	
yeast (<i>Saccharomyces cerevisiae</i>)	-----KIQ	

CHAPTER 4 EGFR MEDIATES ATM TYROSINE PHOSPHORYLATION

4.1 Identification of kinase mediating ATM tyrosine phosphorylation

To determine which tyrosine kinase might be responsible for ATM tyrosine phosphorylation after IR stimulation, we first tested a series of tyrosine kinase inhibitors (TKIs) for their effect on ATM tyrosine phosphorylation by immunoprecipitation (IP) using 4G10, a pan anti-phosphotyrosine antibody. Pretreatment with epidermal growth factor receptor (EGFR) kinase inhibitors (gefitinib and AG1478) significantly decreased ATM tyrosine phosphorylation levels (Figure 5A). In contrast, pretreatments with other TKIs, including imatinib, crizotinib, SU4312, PD173074, masitinib, and picropodophyllin, did not have any substantial effects on ATM tyrosine phosphorylation (Figure 5A). These results suggest that EGFR is a potential tyrosine kinase of ATM.

4.2 ATM is phosphorylated by EGFR *in vitro* and *in vivo*

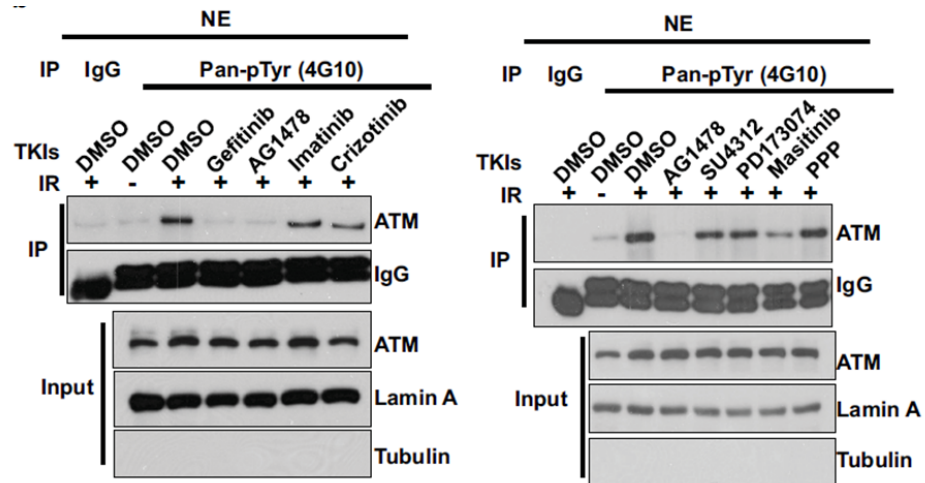
Next, we asked whether EGFR can directly phosphorylate ATM *in vitro*. Indeed, results from *in vitro* kinase assay indicated that EGFR directly phosphorylated ATM (Figure 5B). In addition, purified human EGFR kinase domain directly phosphorylated a recombinant GST-ATM2 fragment (residues 250-522) (Khanna et al., 1998) but not the one containing the

Y370F mutation (Figures 5C). Moreover, we performed co-immunoprecipitation (co-IP) assay in 293T cells and found we can only detect ATM tyrosine phosphorylation when ATM was co-immunoprecipitated with EGFR wild type (Figure 5D). ATM tyrosine phosphorylation signal was reduced when co-IP with EGFR kinase dead (KD) mutant or pretreatment of EGFR kinase inhibitor gefitinib, which strongly suggests that EGFR is a bona fide kinase of ATM.

Figure 5 EGFR mediates ATM tyrosine phosphorylation.

A. Identification of kinase mediating ATM tyrosine phosphorylation.

Western blot analysis of HeLa cells nuclear extracts (NE) pretreated with vehicle (DMSO) or tyrosine kinase inhibitors (TKIs) as indicated, followed by IR stimulation and IP with pan-pTyr antibody (4G10). Lamin A and tubulin served as nuclear and cytosolic markers, respectively. NE indicates nuclear extract. Imatinib, Bcr-Abl kinase inhibitor; crizotinib, lymphoma kinase, c-ros oncogene 1 receptor tyrosine kinase (ROS1), and c-Met inhibitor; SU4312, vascular endothelial growth factor (VEGF) receptor protein tyrosine kinase 1/2 and platelet derived growth factor (PDGF) receptor inhibitor; PD173074, fibroblast growth factor receptor inhibitor; masitinib, stem cell growth factor receptor (c-kit) and platelet-derived growth factor (PDGF) receptor inhibitor; picropodophyllin (PPP), insulin-like growth factor-1 (IGF) receptor inhibitor.



B. EGFR phosphorylates purified Flag-tagged ATM in vitro.

Purified Flag-tagged ATM proteins were incubated with or without purified recombinant human EGFR kinase *in vitro* and analyzed by Western blotting with indicated antibodies.

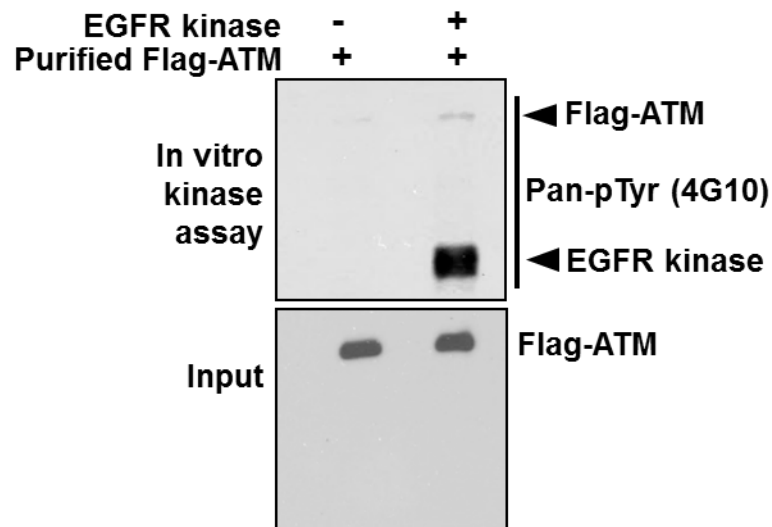


Figure 5 EGFR mediates ATM tyrosine phosphorylation.

- C. EGFR phosphorylates purified recombinant GST-ATM2 fragment but not GST-ATM2/Y370F mutant.

Up panel: schematic of GST only (GST), GST-fused ATM2 and GST-ATM2-Y370F (GST-ATM2/Y370F) constructs. ATM2 indicates ATM residues 250-522.

Bottom panel: recombinant GST, GST-ATM2 and GST-ATM2/Y370F proteins were incubated with purified recombinant human EGFR kinase (His672-Ala1210) *in vitro*, analyzed by SDS-PAGE, and detected by γ - ^{32}P exposure (kinase assay) or Coomassie brilliant blue staining.

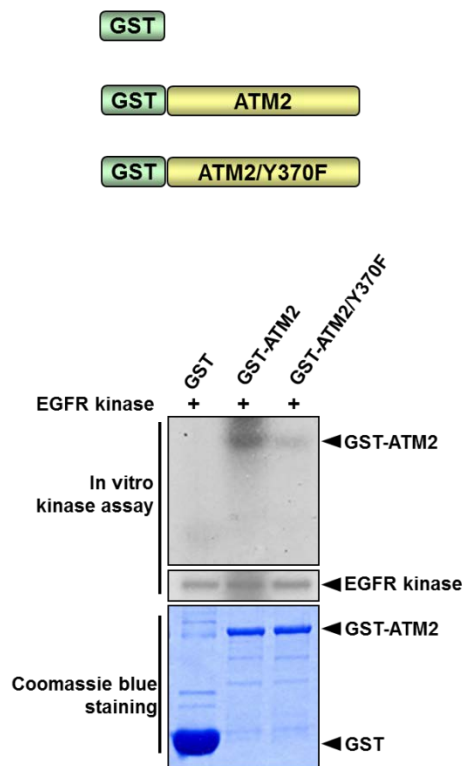
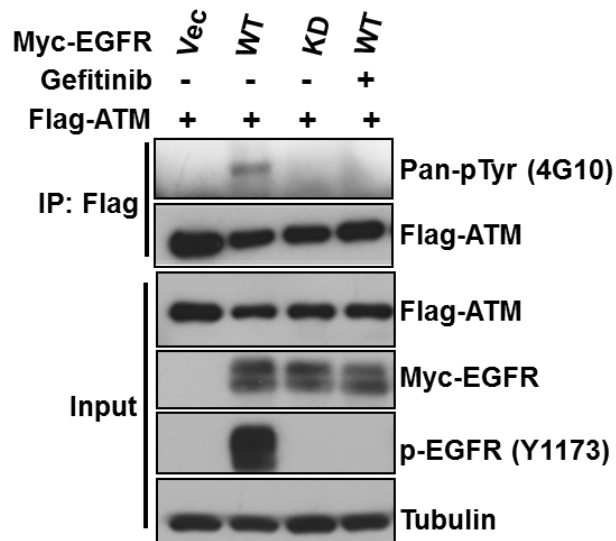


Figure 5 EGFR mediates ATM tyrosine phosphorylation.

D. EGFR kinase activity is required for ATM tyrosine phosphorylation.

HEK 293T cells transfected with the indicated plasmids were treated by IR stimulation. The resulting cells were harvested for co-immunoprecipitation followed by Western blot analysis. Gefitinib: EGFR kinase inhibitor.



CHAPTER 5 EGFR ASSOCIATES WITH ATM IN THE NUCLEUS IN RESPONSE TO IR

5.1 Endogenous EGFR associates with ATM in the nucleus upon IR stimulation.

Previously we found EGFR mediates ATM tyrosine phosphorylation upon IR stimulation, so we hypothesize that EGFR may associate with ATM in response IR. EGFR has been shown to translocate to the nucleus upon various stimuli, including IR (Dittmann et al., 2005a; Dittmann et al., 2008a; Wang and Hung, 2009). We demonstrated that IR not only provoked EGFR nuclear translocation (Figure 6A) but also induced endogenous EGFR association with ATM in the nucleus as determined by immunoprecipitation and reciprocal immunoprecipitation assays using anti-EGFR and anti-ATM antibodies, respectively, against nuclear extracts (Figures 6B and 6C). Meanwhile, pretreatment with gefitinib reduced the interaction between EGFR and ATM (Figures 6B and 6C), suggesting the association between EGFR and ATM depends on EGFR kinase activity.

EGFR is a plasma membrane abundant protein which locates in the non-nuclear compartment of cell. We ask whether EGFR binds to ATM in the cytosolic fraction. As shown in Figure 6D, immunoprecipitation of EGFR

cannot pull down ATM in the cytosolic fraction, suggesting EGFR only interacts with ATM in the nucleus.

5.2 EGFR C-terminal CR domain is required for ATM interaction.

To identify which domain of EGFR is responsible for interaction with ATM, we performed domain mapping of EGFR with ATM by using co-IP analysis. Among various functional domains (Figure 6E, up panel), the C-terminal regulatory region (CR) domain of EGFR seemed to be required for its association with ATM (Figure 6E, bottom panel). Together, these results indicate that EGFR translocates to the nucleus and phosphorylates ATM and that the CR domain of EGFR is required for ATM interaction upon IR stimulation.

Figure 6 Endogenous EGFR associates with ATM in the nucleus upon IR stimulation.

A. EGFR translocates to the nucleus after IR stimulation.

HeLa cells were serum starved, treated with DMSO or gefitinib, and stimulated with or without 10 Gy IR. The resulting cells were harvested for nuclear fractionation, followed by co-immunoprecipitation assays as shown in Figures 6B, 6C and 6D.

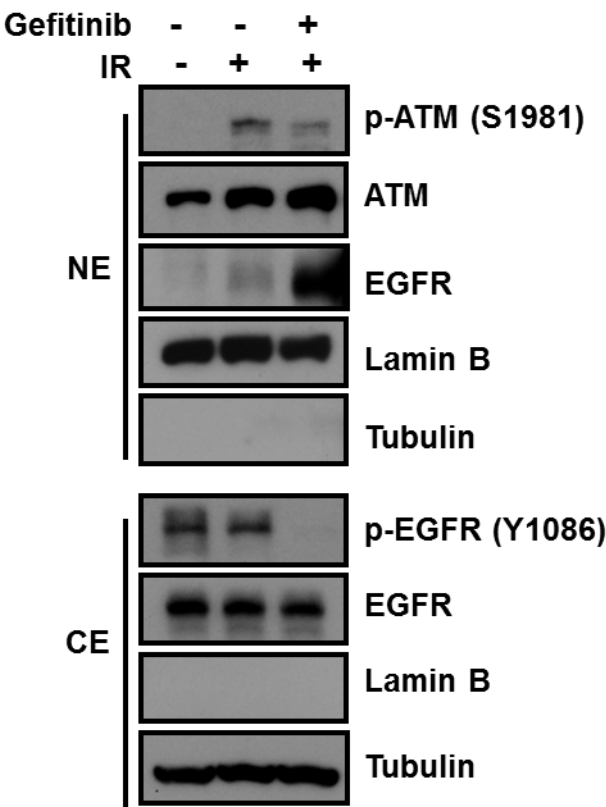
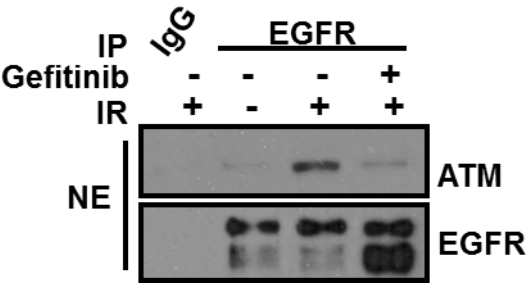


Figure 6 Endogenous EGFR associates with ATM in the nucleus upon IR stimulation.

B. EGFR associates with ATM in the nucleus.

Western blot analyses of endogenous EGFR IP products from NE with indicated antibodies.



C. ATM associates with EGFR in the nucleus.

Western blot analyses of endogenous ATM IP products from NE with indicated antibodies.

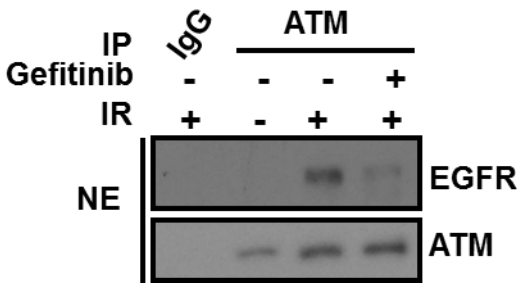
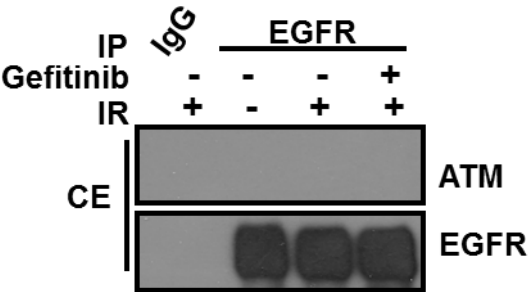


Figure 6 Endogenous EGFR associates with ATM in the nucleus upon IR stimulation.

D. EGFR fails to interact with ATM in the cytosolic compartment of cell.

Western blot analyses of endogenous EGFR IP products from CE with indicated antibodies.

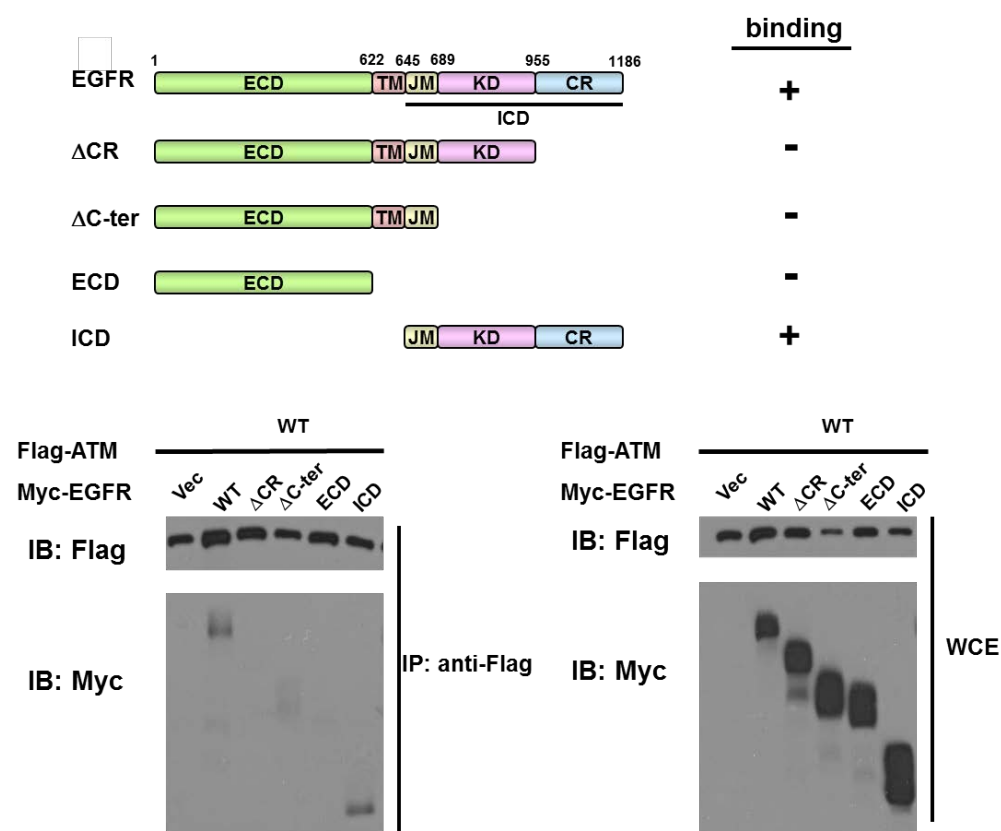


E. EGFR C-terminal CR region is required for ATM interaction.

Up panel: schematic representation of the EGFR domain structure. Myc-tagged EGFR wild type or truncated mutants including catalytic region deletion, C-terminal deletion, intra-cellular domain deletion or extra-cellular domain deletion are designed as WT, Δ CR, Δ C-ter, ECD and ICD, respectively. “+” indicates interaction with ATM. “-” indicates no interaction with ATM.

Bottom panel: HEK 293T cells were transfected with plasmids as indicated and harvested for Co-IP assay followed by Western blot

analysis after IR stimulation. ECD: extracellular domain. TM: transmembrane. JM: juxtamembrane. KD: kinase domain. CR: C-terminal regulatory region. ICD: intracellular domain. IB: immunoblotting. WCE: whole cell extract.



CHAPTER 6 EGFR IS RECRUITED TO DSBs IN RESPONSE TO DNA DAMAGE STIMULI

6.1 EGFR co-localizes with ATM at *I-SceI* induced DSBs.

ATM is known to be recruited by MRN complex to DSBs in response to DSBs formation (Derheimer and Kastan, 2010; Uziel et al., 2003) and we found EGFR associates with and phosphorylated ATM upon IR, implying that EGFR may be recruited to DSBs like ATM. To investigate whether EGFR is recruited to DSBs upon DNA damage stimuli, we first carried out chromatin immunoprecipitation (ChIP) combined with PCR in DR-GFP reporter integrated U2OS cell system (Figure 7A) (Peng et al., 2009). We found that exogenous *I-SceI* expression induced the recruitment of endogenous EGFR to the DSBs (Figure 7B) similar to activated ATM (p-ATM S1981; Figure 7C).

6.2 EGFR is recruited to DSBs in response to laser light activation.

Then, using a KillerRed light activation system (Figure 7D; detailed information is described in Materials and Methods) that generates DSBs, we also showed in Figure 7E that GFP-tagged EGFR signal (indicated by yellow arrowheads in the lower panels) overlapped with tetR-KillerRed (orange color in the merged inset), which was not observed in the control (top panels), further substantiating the localization of EGFR at DSBs upon laser activation.

Together, these data suggest that EGFR, like ATM, is recruited to DSBs upon DNA damage stimulation.

Figure 7 EGFR is recruited to DSBs in response to DNA damage stimuli.

A. Schematic representation of DR-GFP reporter integrated in U2OS cell.

Full-length GFP expression DNA was truncated by I-SceI restriction enzyme recognition sequence (marked as red). With exogenous I-SceI expression-induced DNA DSB, homologous recombination efficiency can be determined by full-length GFP expression by using flow cytometry.

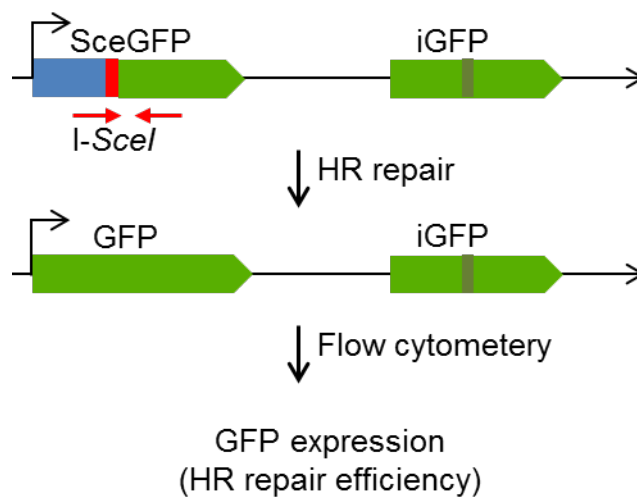


Figure7. EGFR is recruited to DSBs in response to DNA damage stimuli.

B. EGFR is recruited to I-SceI induced DSBs.

ChIP assay was performed with anti-EGFR antibodies or IgG in I-SceI-induced DSBs DR-GFP-integrated U2OS cells. Specific primers (marked as red arrows in Figure 7A) flanking I-SceI site were used in PCR to detect activated ATM and EGFR localized at DSBs. Quantitation of DSB recruitment fold change is presented as mean \pm SD. $n = 3$. $*p < 0.05$.

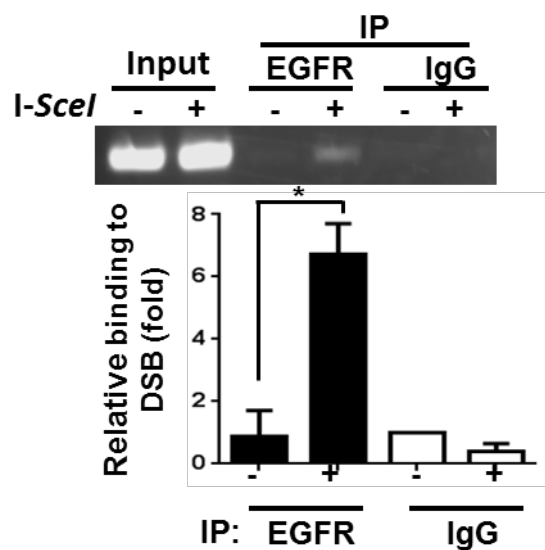


Figure7 EGFR is recruited to DSBs in response to DNA damage stimuli.

C. Activated ATM is recruited to I-SceI induced DSBs.

ChIP assay was performed with p-ATM S1981 (pATM) or IgG in I-SceI-induced DSBs DR-GFP-integrated U2OS cells. Specific primers (marked as red arrows in Figure 7A) flanking I-SceI site were used in PCR to detect activated ATM and EGFR localized at DSBs. Quantitation of DSB recruitment fold change is presented as mean \pm SD. $n = 3$. $*p < 0.05$.

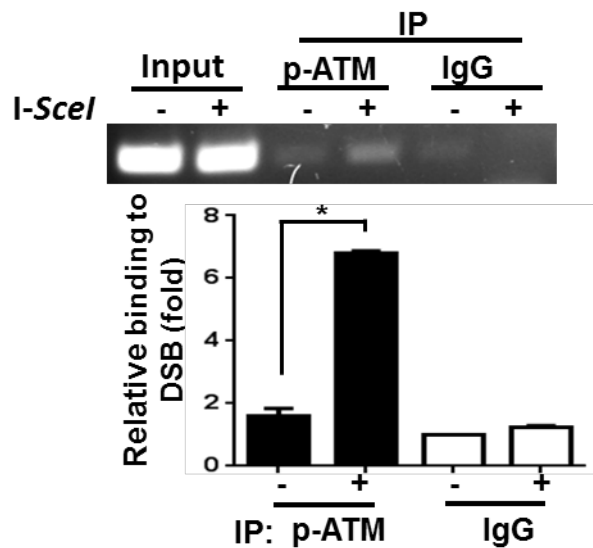


Figure7 EGFR is recruited to DSBs in response to DNA damage stimuli.

D. Schematic representation of the KillerRed system in U2OS TRE cell.

KillerRed is a light-stimulated ROS-inducer fused to a tet-repressor (tetR-KR), which binds to a TRE cassette (~ 90 kb) integrated at a defined genomic locus in U2OS cells (U2OS TRE cell line) (Lan et al., 2013; Lan et al., 2010). KR facilitates the formation of oxygen radicals and superoxide through the excited chromophore (Carpentier et al., 2009; Pletnev et al., 2009) to induce DNA damage. By targeting the expression of KR to one specific genome site, we can visualize the recruitment of proteins at genetic loci.

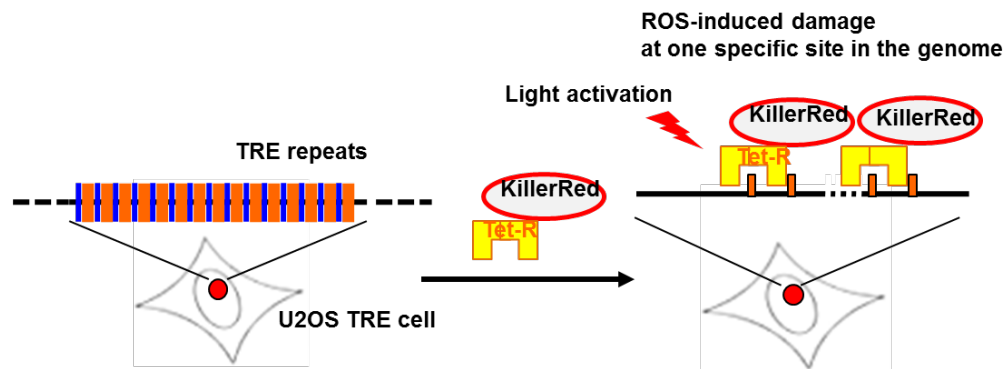
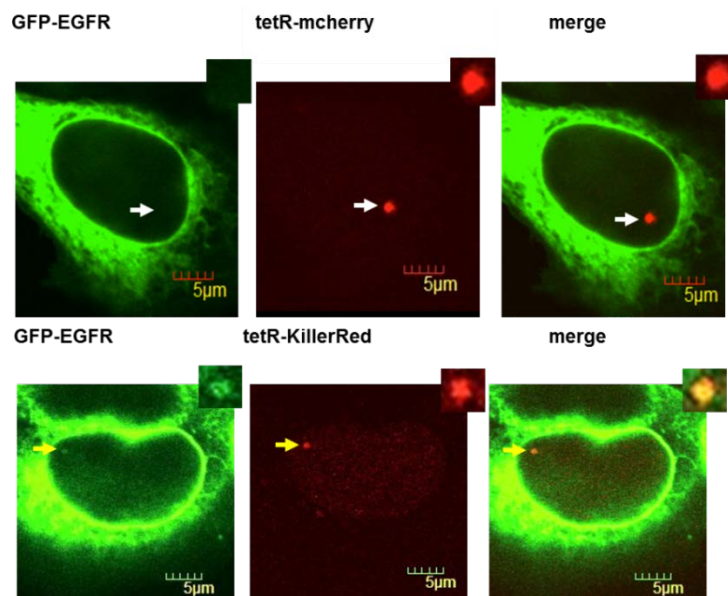


Figure7 EGFR is recruited to DSBs in response to DNA damage stimuli.

E. GFP-tagged EGFR is recruited to DSBs in response to laser light.

GFP-tagged EGFR and tetR-mcherry or tetR-KR were transfected into U2OS TRE cells. The KillerRed spot was activated with 559 nm laser to induce DNA damage. Representative images after DNA damage induced by KillerRed activation are shown in the lower panels. White arrowheads (also shown in enlarged insets): a tet-repressor fused monomer cherry (tetR-mcherry) binds to a TRE cassette integrated at a defined genomic locus in U2OS cells without laser light activated DNA damage. Yellow arrowheads (also shown in enlarged insets): DNA damage sites induced by a tet-repressor fused KillerRed (tetR-KR) expression and light activation as described in Figure 7D.



CHAPTER 7 EGFR CO-LOCALIZES WITH ATM AND γ -H2AX AT DSBs

7.1 EGFR co-localizes with ATM and γ -H2AX at laser irradiation-induced DSBs.

To examine whether EGFR co-localizes with ATM or other DDR proteins such as γ -H2AX at DSBs, we treated U2OS or HeLa cells by laser irradiation (Lan et al., 2005; Lan et al., 2004) and found overlapping signals indicative of co-localization between GFP-tagged (yellow arrowheads, Figure 8A) or endogenous EGFR (yellow arrowheads or enlarged insets, Figure 8B) and activated ATM (p-ATM S1981) or γ -H2AX (Figure 8C). The results suggested both endogenous and GFP-tagged EGFR are able to be recruited to and co-localize with ATM or γ -H2AX at DSBs.

7.2 EGFR associates with MRN complex in the nucleus.

As ATM is recruited to DSBs by Mrell-Rad50-NBS1 (MRN) complex (Uziel et al., 2003) and activated by MRN *in vitro* (Lee and Paull, 2004, 2005), we suspected whether EGFR can also participate into ATM/MRN complex at DSBs. To this end, we showed that EGFR immunoprecipitation can pull down the MRN complex in the nuclear extract after IR treatment (Figure 8D). These results suggest that EGFR may play a role in DDR by associating with ATM and the MRN complex at DSBs upon IR stimulation.

Figure 8 EGFR co-localizes with ATM and other DDR proteins at DSBs.

A. GFP-tagged EGFR co-localizes with ATM at laser irradiation-induced DSBs.

GFP-tagged EGFR-transfected U2OS cells were irradiated with 405 nm for 100 ms. After irradiation, cells were fixed and stained with p-ATM S1981 antibody. Laser microirradiation-induced DSBs is indicated by yellow arrowheads.

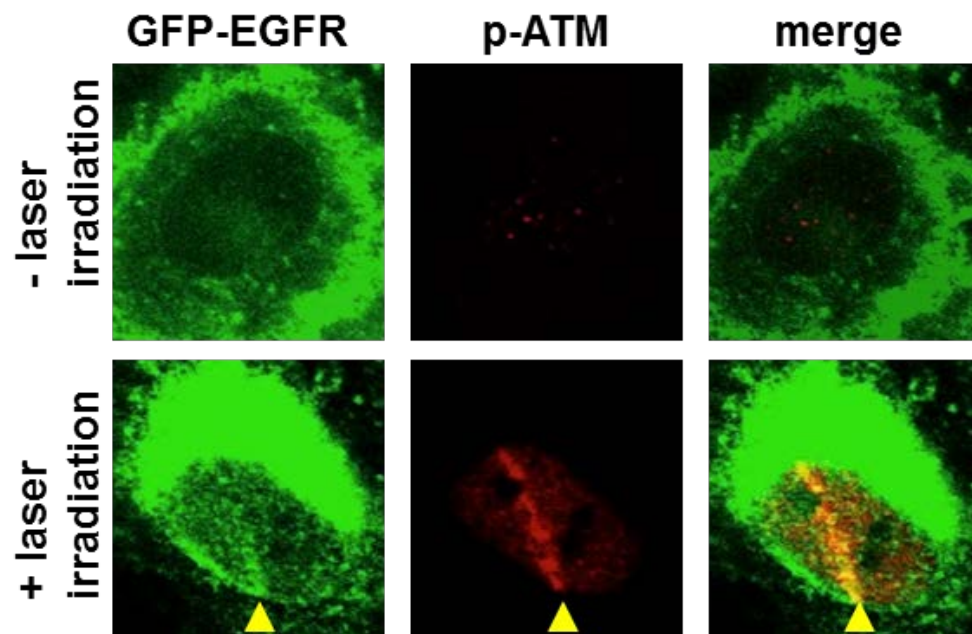


Figure 8 EGFR co-localizes with ATM and other DDR proteins at DSBs.

B. Endogenous EGFR co-localizes with ATM at laser irradiation-induced DSBs.

HeLa cells are irradiated with 405 nm for 60 ms. After irradiation, cells were fixed and stained with antibodies against EGFR and p-ATM S1981. Laser irradiation-induced DSBs are indicated by yellow arrowheads (also shown in enlarged insets).

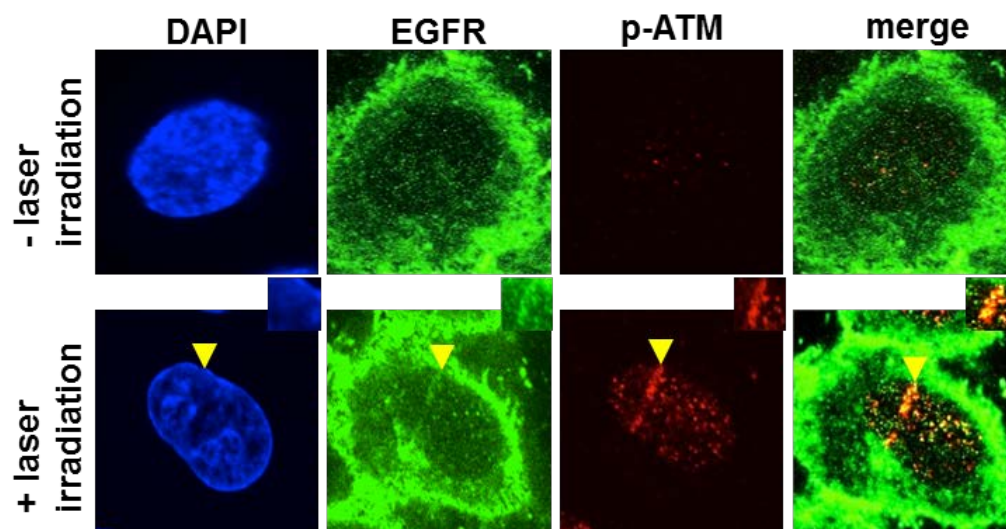


Figure 8 EGFR co-localizes with ATM and other DDR proteins at DSBs.

C. GFP-tagged EGFR co-localizes with γ -H2AX at micro-laser IR induced DSBs.

GFP-EGFR transfected U2OS cells are irradiated with 405 nm for 100 ms. Fifteen minutes after laser irradiation, cells are fixed and stained with indicated antibody. Yellow arrowheads, laser microirradiation-induced DSBs.

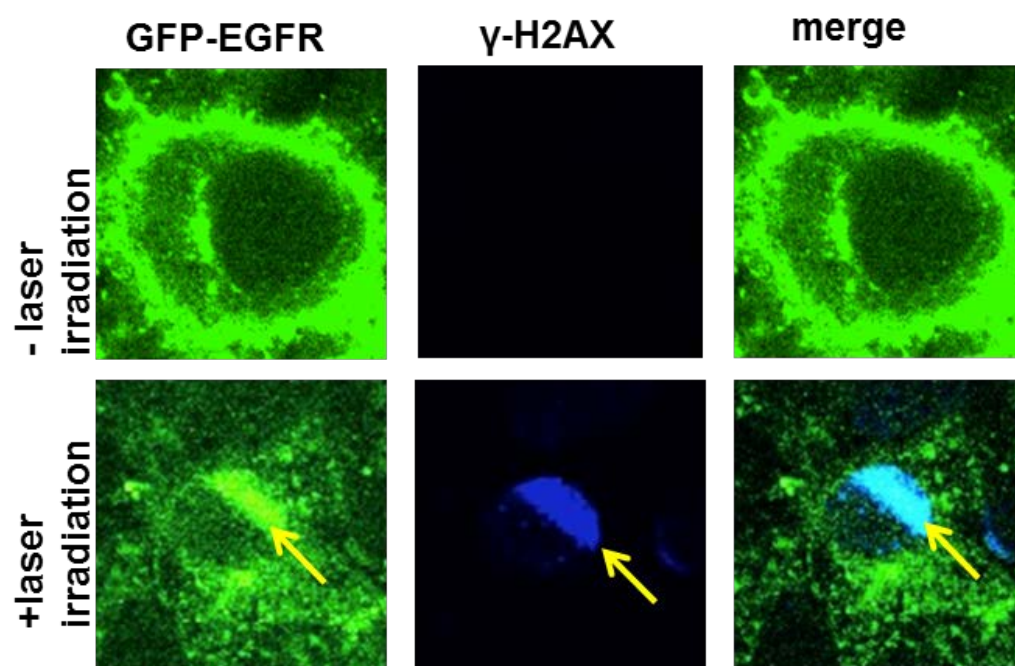
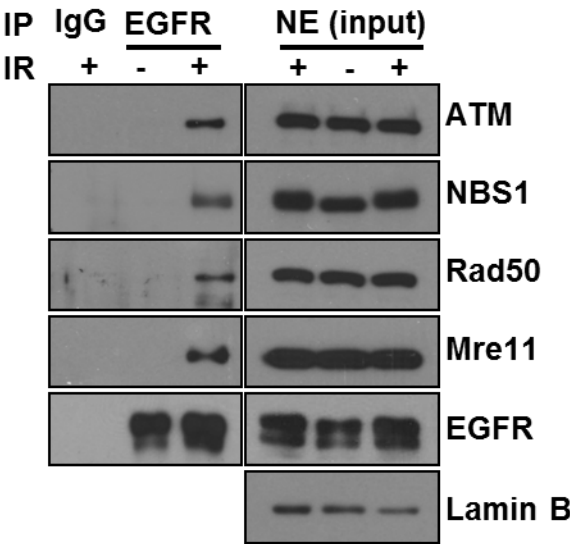


Figure 8 EGFR co-localizes with ATM and other DDR proteins at DSBs.

D. Nuclear EGFR associates with MRN complex after IR stimulation.

Western blotting analysis of endogenous EGFR IP products from NE treated with or without IR. NE: nuclear extract. Lamin B: nuclear fraction marker.



CHAPTER 8 EGFR IS REQUIRED FOR ATM AND CHK2 IRIF

8.1 EGFR is required for ATM IRIF

As we demonstrated that EGFR phosphorylates and interacts with ATM upon IR, we suspect that EGFR may participate into DDR like ATM and other DDR proteins. First, we aimed to determine whether EGFR is required for ATM downstream functions, such as irradiation-induced foci formation (IRIF) and DNA repair ability, we generated EGFR knockdown pooled stable clones using lentiviral-based shRNA targeted against EGFR (up panel, Figure 9A). Silencing EGFR impaired p-ATM S1981 IRIF (bottom panel, Figure 9A) and chromatin-enriched fractionation also showed that ATM S1981 phosphorylation levels were lower in EGFR knockdown stable clones from two different shRNAs than control cells (Figure 9B). Importantly, pretreatment with EGFR kinase inhibitor gefitinib also abolished p-ATM S1981 IRIF induced by laser microirradiation (Figure 9C), suggesting EGFR indeed required for ATM IRIF.

8.2 Depletion of EGFR impairs CHK2 IRIF

CHK2 is one of the major ATM downstream kinase which phosphorylates other DDR proteins and mediates cell cycle checkpoints after being activated and phosphorylated by ATM at serine 68 (Ward et al., 2001).

To test whether EGFR involves in ATM downstream signaling, we found depletion of endogenous EGFR impairs CHK2 IRIF (Figure 9D). Together, these results indicated that EGFR and its kinase activity are required for ATM and CHK2 IRIF in DDR.

Figure 9 EGFR is required for ATM and CHK2 IRIF

A. EGFR is required for ATM IRIF.

Up panel: Western blot analysis of control or two different EGFR-knockdown U2OS cells. Bottom panel: Immunofluorescent (IF) staining of irradiation-induced foci (IRIF) of control or EGFR-depleted (shEGFR) U2OS cells with indicated antibodies. Quantitation of p-ATM S1981 and p-CHK2 IRIF is presented as mean \pm SD. $n=103$. $*p < 0.05$.

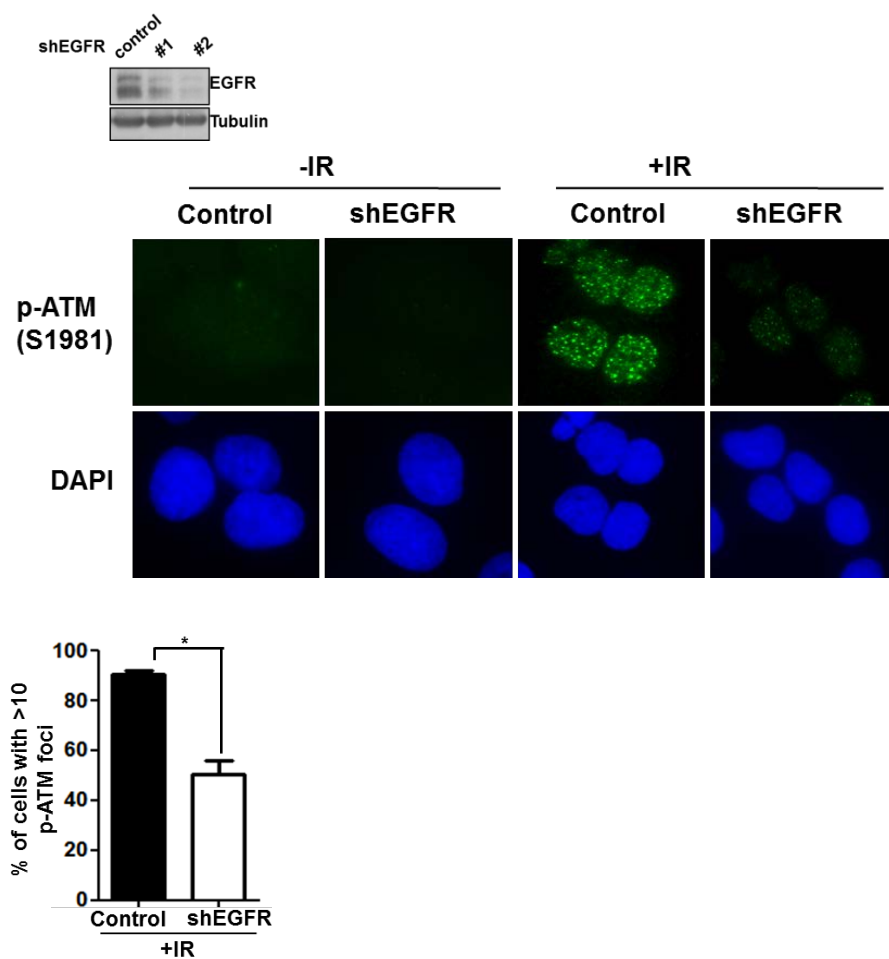


Figure 9 EGFR is required for ATM and CHK2 IRIF

B. Knocking down endogenous EGFR decreases ATM pS1981 level in chromatin enrich fractionation.

Chromatin enriched fractionation and WCE of control or EGFR-knockdown HeLa cells stimulated with or without IR. Control: shRNA vector control. #1 and #2 indicate two different clones of shEGFR. WCE: whole cell extract. ORC2: origin replication complex 2, serving as a chromatin fraction marker. Tubulin: cytosolic marker.

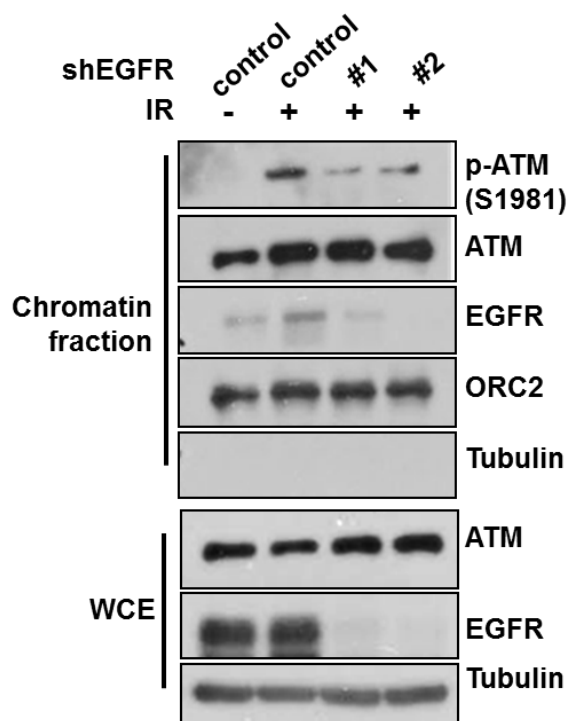


Figure 9 EGFR is required for ATM and CHK2 IRIF

C. EGFR kinase activity is required for ATM IRIF.

U2OS cells are treated with or without 10 μ M Gefitinib for 16 h and irradiated with 405 nm for 100 ms. 15 min after irradiation, cells are fixed and stained with indicated antibody. DAPI: 4,6-diamidino-2-phenylindole. Yellow arrowheads, laser microirradiation-induced DSBs.

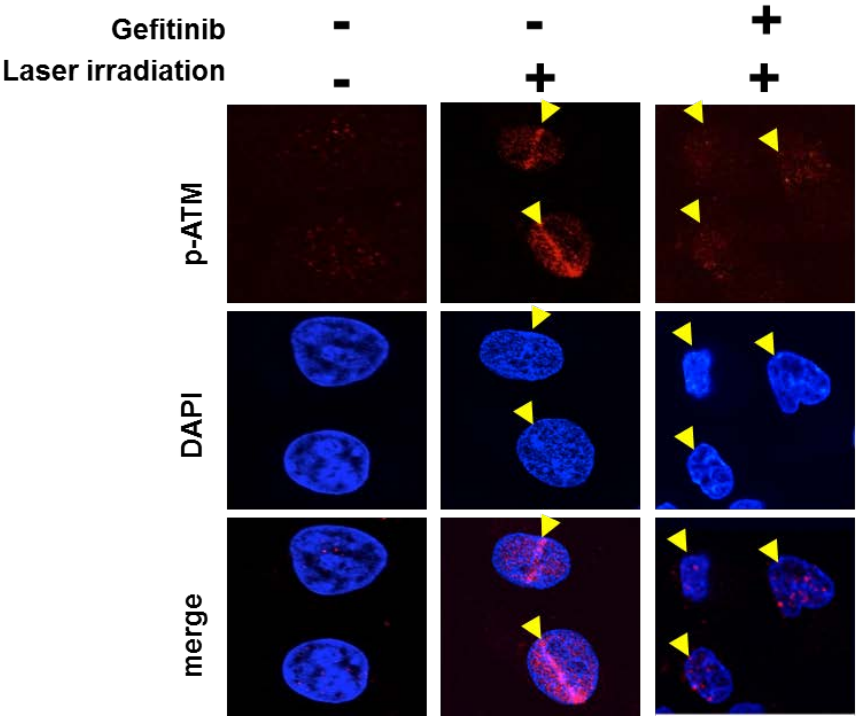
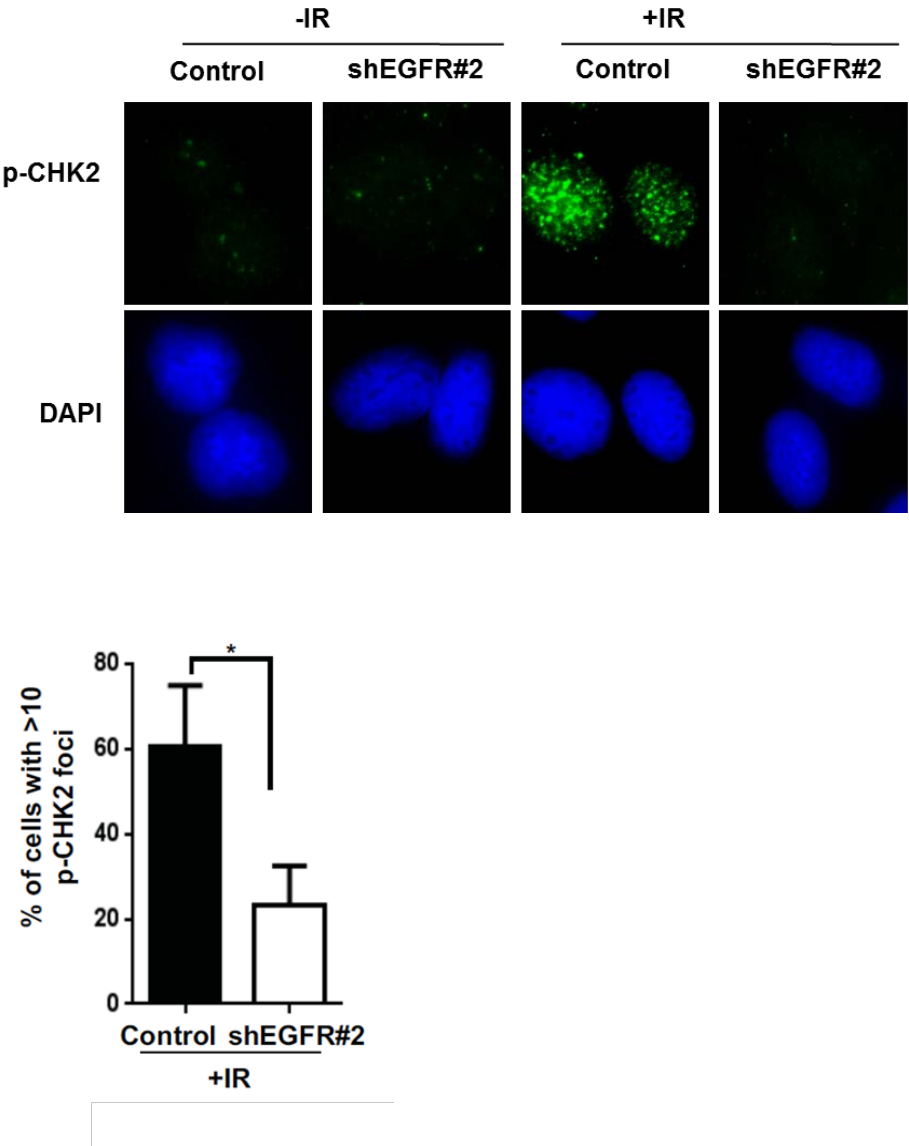


Figure 9 EGFR is required for ATM and CHK2 IRIF

D. Depletion of EGFR impairs CHK2 IRIF.

Immunofluorescent (IF) staining of irradiation-induced foci (IRIF) of control or EGFR-depleted (shEGFR) U2OS cells with indicated antibodies. DAPI: 4,6-diamidido-2-phenylindole.



CHAPTER 9 EGFR IS REQUIRED FOR ATM-MEDIATED DNA REPAIR

9.1 Knocking down-EGFR reduces DNA repair ability.

IRIF of p-ATM S1981 is known to activate or recruit DDR proteins such as NBS1 and BRCA1 to DSBs to execute DNA repair (Cortez et al., 1999; Kastan and Lim, 2000; Lukas et al., 2011). We next established the link between EGFR and DNA repair by carrying out a neutral comet assay, which detects DNA damage level as indicated by comet tail movement. The results showed that EGFR-silenced cells had four times higher DNA damage levels than the control-silenced cells, suggesting that EGFR deficiency reduces DNA repair ability (Figure 10A). To substantiate this finding, we compared the number of EGFR-knockdown and control cells that contained γ -H2AX foci as previous reports have indicated that the kinetics of γ -H2AX foci clearance correlates with mammalian cell radio-sensitivity (MacPhail et al., 2003; Taneja et al., 2004). Indeed, EGFR-depleted cells demonstrated delayed DNA repair efficiency as indicated by a higher percentage of cells containing γ -H2AX foci compared with control cells at 24 and 48 h after IR stimulation (Figure 10B).

9.2 EGFR is required for ATM-mediated homologous recombination repair.

To further determine whether EGFR is involved in ATM-mediated homologous recombination repair (Iijima et al., 2008; Morrison et al., 2000), we generated control and EGFR-depleted U2OS cells integrated with DR-GFP reporter (Figure 7A) (Peng et al., 2009). We found that DSBs induced by exogenous I-SceI expression was efficiently repaired, as indicated by the number of GFP-expressing cells, in control but not in EGFR-depleted cells (Figure 10C). All together, these results suggest that EGFR is required for ATM-mediated IRIF and DNA repair.

Figure 10 EGFR involves in DNA repair.

A. Depletion of EGFR impairs DNA repair.

Comet assay of EGFR-depleted HeLa cells were carried out with or without 15 Gy IR. Top: four representative figures showing comet tail movements (indicated by red bars) in EGFR knockdown or vector control HeLa cells after IR treatment 6 hours. Bottom: quantitation from three independent experiments with or without IR exposure. Cells were measured by CometScore software in each experiment. $n=50$. $**p < 0.01$. Western blot analysis of control or EGFR-knockdown HeLa cells used in comet assay. Control: EGFR shRNA vector control.

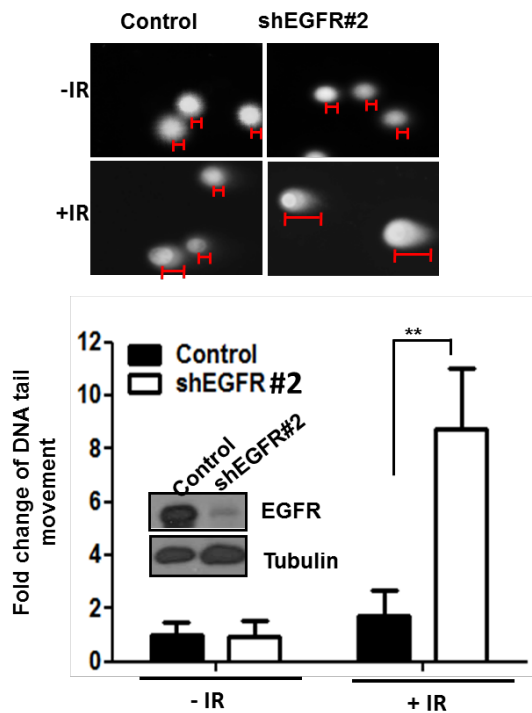


Figure 10 EGFR involves in DNA repair.

B. Knocking-down endogenous EGFR delays DNA repair.

Quantification of the percentage of cells with an amount of γ -H2AX foci after IR in control (shCtrl) or two EGFR-depleted (shEGFR#1 and #2) U2OS cells. Cells were exposed to 5 Gy of ionizing radiation (IR), fixed after 0, 4 hours, 24 hours and 48 hours and stained with DAPI and antibodies against γ -H2AX. Percentage of γ -H2AX foci staining positive cells was quantitated among various fields. $**p < 0.01$.

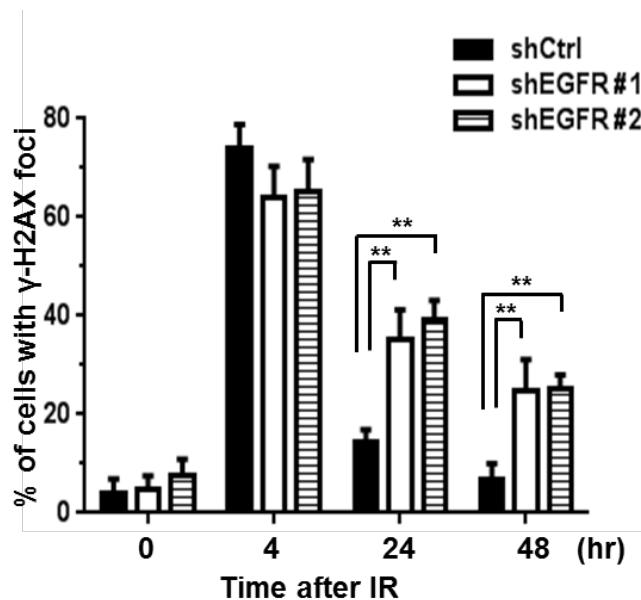
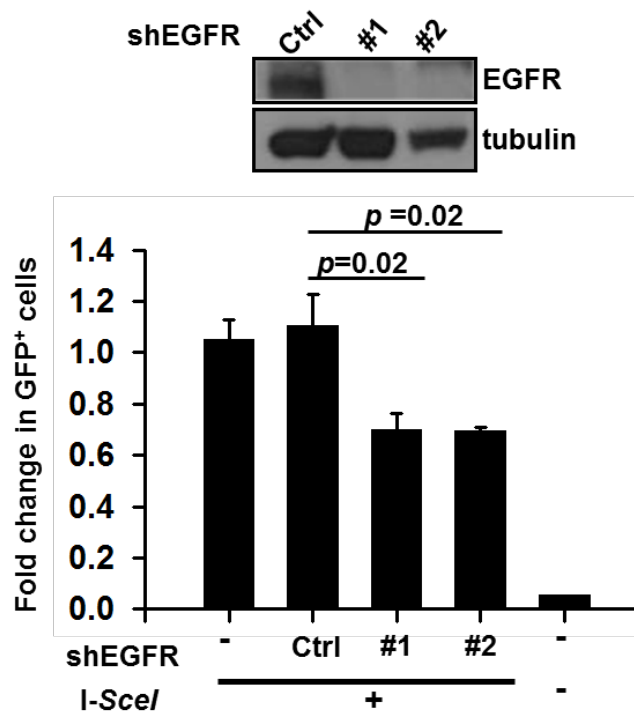


Figure 10 EGFR involves in DNA repair.

C. EGFR is required for ATM-mediated homologous recombination repair.

Homologous recombination efficiency in DR-GFP reporter-integrated U2OS cells with control or EGFR silencing (shEGFR #1 and #2) was determined by flow cytometry after ectopic expression of *I-SceI*. Top: Western blotting showing two EGFR-targeting shRNA #1 and #2 knockdown efficiency in DR-GFP integrated U2OS cells. Bottom: quantitation of three independent experiments. Ctrl: EGFR shRNA vector control. * $p = 0.02$.



CHAPTER 10 EGFR MEDIATES ATM Y370 PHOSPHORYLATION

10.1 ATM Y370 is a major EGFR-mediated phosphorylation site.

Next, we asked if EGFR phosphorylates ATM at the conserved Y370 and mediates ATM function through this phosphorylation event. Co-immunoprecipitation of Myc-tagged EGFR with Flag-tagged ATM in HEK293T cells showed that ectopic expression of wild-type (WT) but not kinase-dead (KD) mutant EGFR enhanced ATM tyrosine phosphorylation *in vivo* (Figure 11A). Consistently with the above finding in which gefitinib pretreatment reduced the association between endogenous EGFR and ATM (Figures 6B and 6C), we found that only EGFR WT but not KD mutant interacted with Flag-tagged ATM (Figure 11A). This suggests that the kinase activity of EGFR is required for its binding with ATM. In addition, the ATM Y370F mutant decreased its ability to bind to EGFR as well as its levels of tyrosine phosphorylation, further supporting that Y370 serves a major EGFR phosphorylation site.

10.2 Generation of AYM pY370 antibody.

To investigate the role of Y370 phosphorylation *in vivo*, we generated a specific antibody against ATM Y370 phosphorylation and validated the specificity of this antibody in which only the phospho-Y370 peptide but not

the non-phospho-Y370 peptide or other phospho-Y peptides was recognized (Figure 11B). Using this antibody, we showed that phospho-Y370 level was decreased when Flag-tagged Y370F but not WT was co-immunoprecipitated with Myc-tagged EGFR (Figure 11C). Moreover, the levels of phospho-Y370 increased upon IR but reduced when cells were pretreated with gefitinib (Figure 11D), suggesting that IR-induced ATM Y370 phosphorylation relies on EGFR's kinase activity.

Figure 11 ATM Y370 phosphorylation is mediated by EGFR

A. ATM Y370 serves as a major EGFR phosphorylation site.

HEK 293T cells transfected with the indicated plasmids were treated by IR stimulation. The resulting cells were harvested for co-immunoprecipitation followed by Western blot analysis.

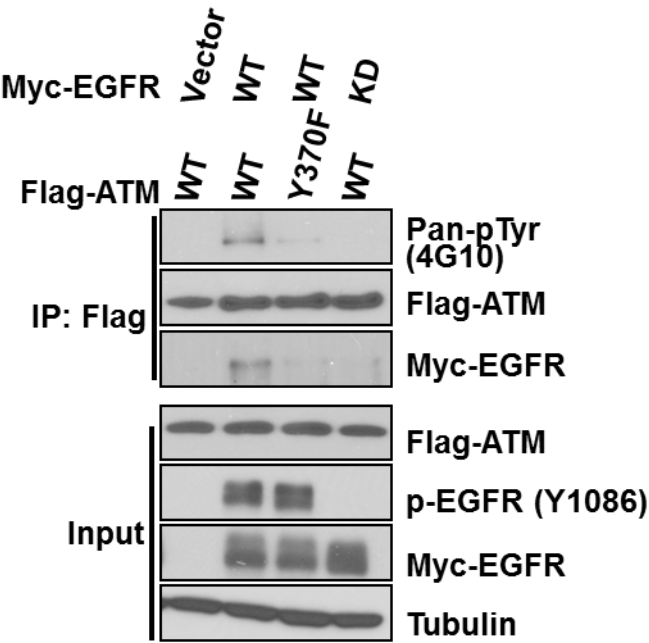


Figure 11 ATM Y370 phosphorylation is mediated by EGFR

B. Characterization of ATM Y370 phosphorylation antibody by using dot blotting.

Various amounts of peptides were spotted on PVDF membrane and detected by p-ATM Y370 antibody or amido black staining. IB: immuno-blotting. ATM pY370: ATM phospho-Y370 peptide. ATM Y370: ATM non-phospho-Y370 peptide. MCM7 pY600: MCM7 phospho-600 peptide. Lyn pY32: Lyn phosphor-32 peptide (Huang et al., 2013).

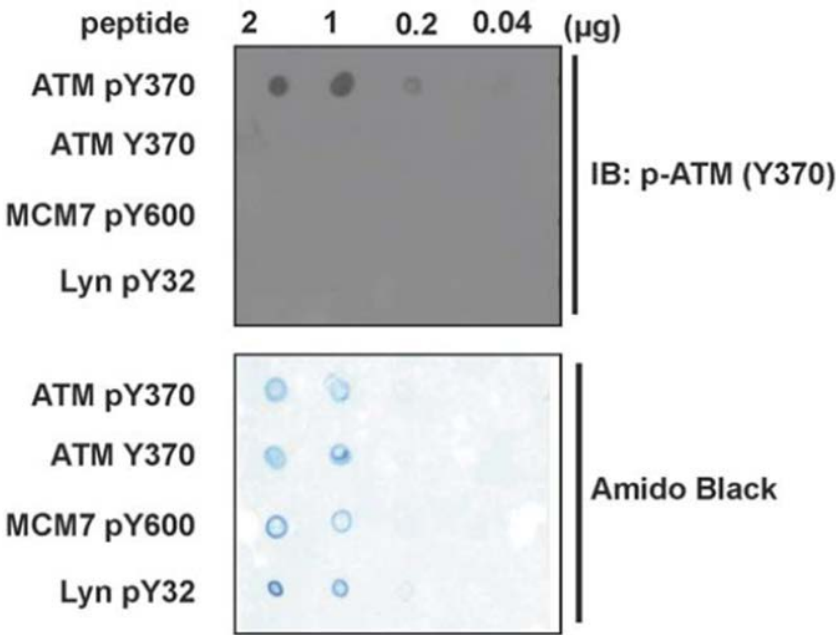


Figure 11 ATM Y370 phosphorylation is mediated by EGFR

C. Characterization of ATM Y370 phosphorylation antibody by western blotting.

HEK 293T cells were transfected with plasmids as indicated and harvested for Co-IP assay followed by Western blot analysis after IR stimulation.

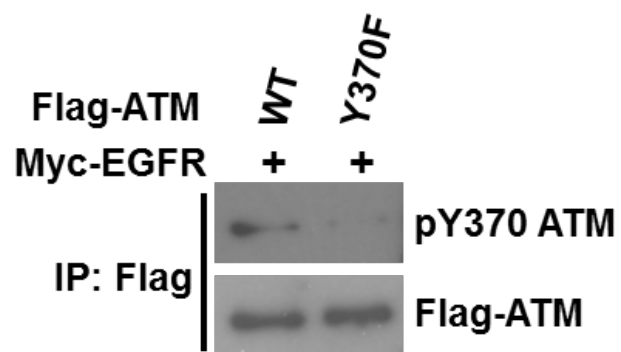
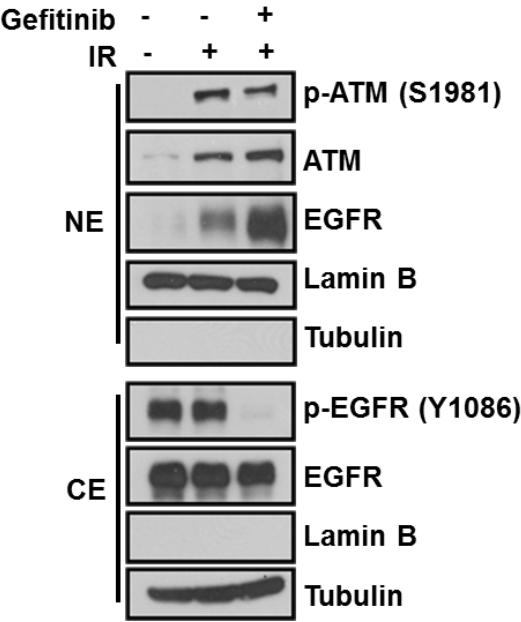
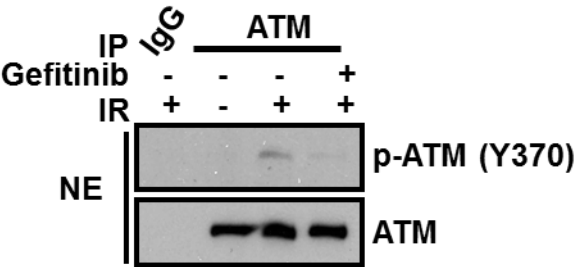


Figure 11 ATM Y370 phosphorylation is mediated by EGFR

D. Endogenous ATM is phosphorylated at Y370 upon IR.

Top: Western blot analysis of endogenous ATM IP products from HeLa cell NE treated with or without IR. Gefitinib: EGFR kinase activity inhibitor. Bottom: western blot analysis of nuclear fractionation.



CHAPTER 11 EGFR MEDIATES DDR via ATM Y370

PHOSPHORYLATION

11.1 ATM Y370 phosphorylation facilitates ATM IRIF.

To validate that ATM Y370 phosphorylation responds to IR and orchestrates DDR, Flag-tagged ATM WT or Y370F mutant was restored in ATM-depleted HeLa cells to examine its effect on p-ATM S1981 IRIF. As shown in Figure 12A, re-expression of ATM WT but not of Y370F mutant rescued p-ATM S1981 IRIF. In addition, compared with ATM WT, recruitment of ATM Y370F to chromatin induced by IR was significantly reduced as demonstrated by chromatin-enriched cell fractionation assay (Figure 12B). These findings suggest that EGFR-mediated ATM Y370 phosphorylation facilitates p-ATM S1981 IRIF.

11.2 ATM Y370 phosphorylation defective mutant impairs downstream CHK2 IRIF and NBS1 S343 phosphorylation.

Activated ATM is known to rapidly phosphorylate protein kinase CHK2 at threonine 68 upon IR stimulation to regulate cell cycle arrest (Ahn et al., 2000; Matsuoka et al., 2000; Ward et al., 2001; Zhou et al., 2000). To decipher whether ATM Y370 phosphorylation plays a role in mediating downstream DDR proteins like CHK2, we examined p-CHK2 IRIF in ATM-

depleted HeLa cells. Re-expression of Flag-tagged ATM WT but not Y370F mutant rescued p-CHK2 IRIF (Figure 12C), indicating that ATM Y370 phosphorylation is also involved in p-CHK2 IRIF. We further investigated whether ATM Y370 phosphorylation is also involved S343 phosphorylation of NBS1, as ATM serves as an upstream kinase in the ATM-NBS1-SMC1 signaling for cell cycle checkpoint (Kitagawa et al., 2004; Lim et al., 2000). Only Flag-tagged ATM WT but not Y370F in ATM-depleted HeLa cells reactivated NBS1 S343 phosphorylation (Figure 12D).

11.3 EGFR regulates DNA-PKcs activation through ATM Y370 phosphorylation.

Emerging data demonstrated DNA-PKcs activation depends on association with nuclear EGFR but detailed mechanism is not clear (Bandyopadhyay et al., 1998; Dittmann et al., 2005a; Dittmann et al., 2005b). A recent report indicated that DNA-PKcs activation relies on ATM S1981 phosphorylation (Chen et al., 2007). We found that restoration of Flag-tagged ATM WT, but not Y370F can rescue DNA-PKcs IRIF, suggesting EGFR regulates DNA-PKcs via ATM Y370 phosphorylation (Figure 12E). Together, these results suggest that EGFR-mediated ATM Y370 phosphorylation is essential for ATM activation, downstream CHK2 and DNA-PKcs IRIF and NBS1 S343 phosphorylation.

Figure 12 EGFR mediated ATM Y370 phosphorylation facilitates ATM IRIF and downstream signaling

A. ATM Y370 phosphorylation facilitates ATM IRIF.

p-ATM S1981 IRIF staining of ATM-depleted (shATM) HeLa cells with reconstitution of Flag-tagged ATM wild type (WT) or Y370F mutant. Quantitation of p-ATM S1981 IRIF is presented as mean values \pm SD. $n=50$. $**p < 0.01$.

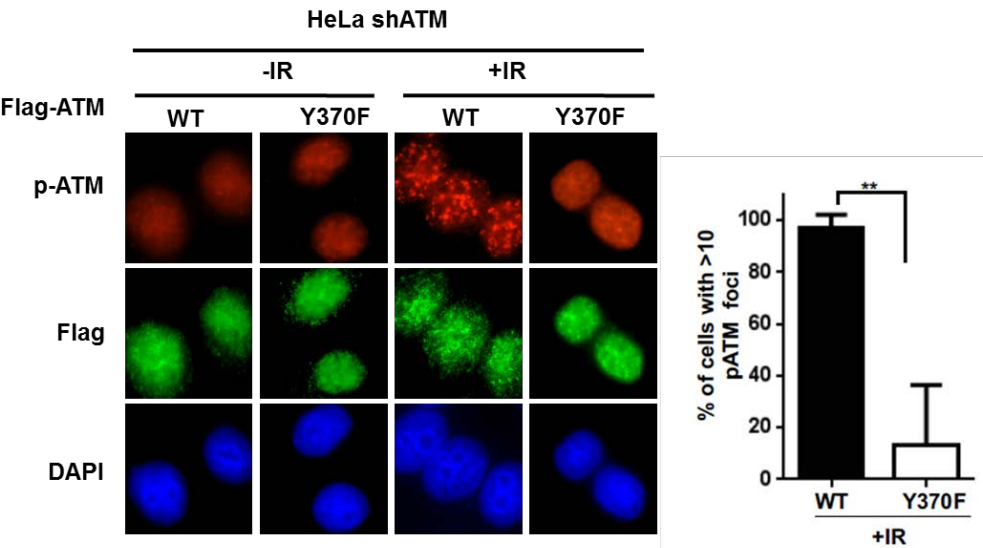


Figure 12 EGFR mediated ATM Y370 phosphorylation facilitates ATM

IRIF and downstream signaling

B. ATM Y370 phosphorylation defective mutant impairs ATM chromatin recruitment.

Vector control, Flag-tagged ATM WT, or Y370F was restored in ATM-depleted HeLa cells (HeLa shATM). After treatment with or without IR and recovery for 4 hours, chromatin enriched fractionation was carried out, followed by Western blot analysis with indicated antibodies.

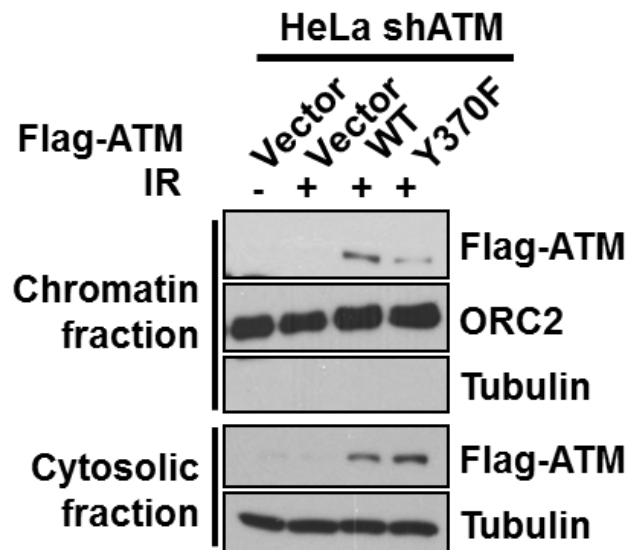


Figure 12 EGFR mediated ATM Y370 phosphorylation facilitates ATM IRIF and downstream signaling

C. ATM Y370 phosphorylation involves in CHK2 IRIF.

p-CHK2 IRIF staining of ATM-depleted HeLa cells with reconstituted Flag-tagged ATM WT or Y370F mutant. Quantitation of p-CHK2 IRIF is presented as mean values \pm SD. $n=50$. $**p < 0.01$.

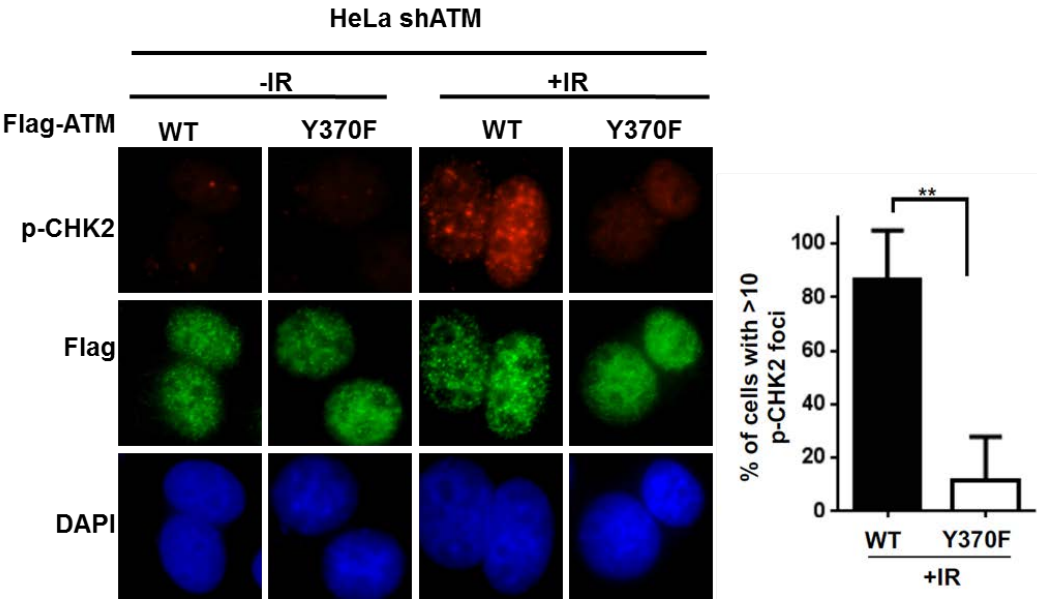


Figure 12 EGFR mediated ATM Y370 phosphorylation facilitates ATM

IRIF and downstream signaling

D. ATM Y370 phosphorylation defective mutant reduces ATM-mediated NBS1 S343 phosphorylation.

ATM-depleted HeLa cells were transfected with Flag-tagged ATM WT, Y370F, or vector alone. Cell transfectants were treated with or without IR, harvested and analyzed by Western blot with indicated antibody.

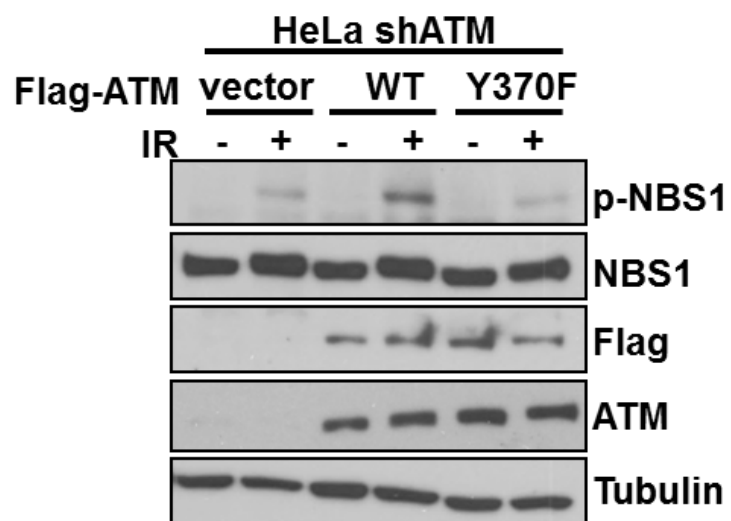
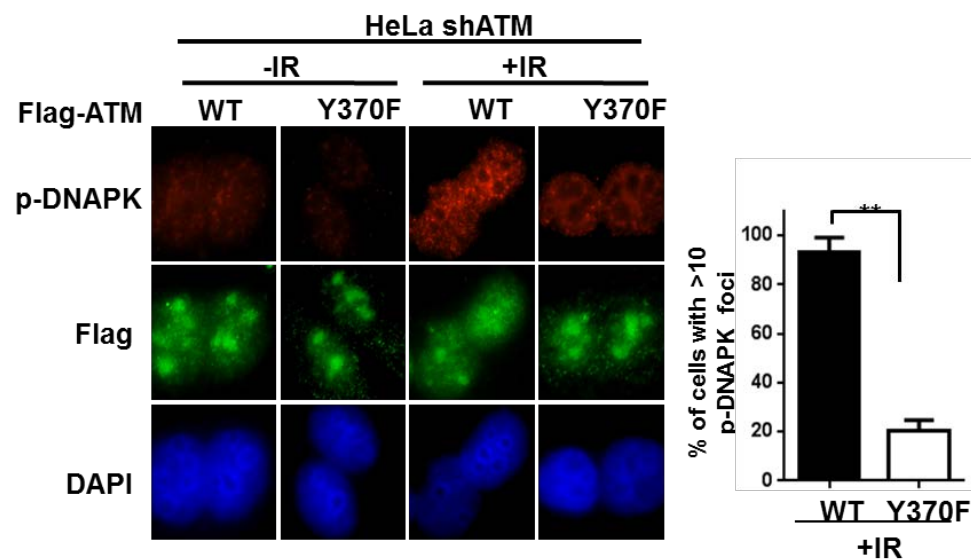


Figure 12 EGFR mediated ATM Y370 phosphorylation facilitates ATM IRIF and downstream signaling

E. ATM Y370 phosphorylation rescues DNA-PKcs IRIF.

DNA-PKcs IRIF staining of ATM-depleted HeLa cells with reconstituted Flag-tagged ATM WT or Y370F mutant. Quantitation of p-DNA-PKcs IRIF is presented as mean values \pm SD. $n = 50$. $**p < 0.01$.



CHAPTER 12 EGFR MEDIATES RADIO-THERAPY RESISTANCE THROUGH ATM Y370 PHOSPHORYLATION

12.1 ATM Y370 phosphorylation is essential for ATM-mediated radio-therapy resistance.

Clinically, EGFR inhibitors synergistically sensitize response to radiation therapy in patients with head and neck squamous cell carcinoma (Bernier et al., 2009). Since EGFR-mediated ATM Y370 phosphorylation is required for ATM activation, downstream signaling, and DNA repair, we hypothesized that ATM Y370 phosphorylation plays a role in radiotherapy resistance. Indeed, HeLa cell colony formation was reduced in ATM-depleted cells compared to that in control cells after IR stimulation but was rescued by reconstitution of only ATM WT but not the Y370F mutant (Figures 13A and 13B), suggesting ATM Y370 phosphorylation is essential for ATM-mediated DNA repair and radiotherapy resistance.

12.2 ATM-mediated radio-therapy resistance depends on EGFR kinase activity.

To show that EGFR regulates radiotherapy resistance through EGFR's kinase activity, we performed colony formation assay by using various doses of EGFR kinase inhibitor gefitinib. Consistent with previous findings (Kang et

al., 2012; Lin et al., 2011; Park et al., 2010), gefitinib combined with IR also produced a synergistic effect in radio-sensitivity (Figure 13C). Collectively, our findings uncovered an underlying mechanism by which gefitinib enhances radio-sensitivity through EGFR-mediated ATM Y370 phosphorylation to facilitate ATM activation and subsequent DDR.

Figure 13 ATM Y370 phosphorylation regulates radio-sensitivity.

A. ATM Y370 phosphorylation is essential for ATM-mediated radio-sensitivity.

ATM-depleted HeLa cells were reconstituted with vector control, Flag-tagged ATM WT, or Y370F and treated with increasing doses of IR. The cell survival rate was determined by colony formation assay and presented as mean value \pm SD. $n = 3$. * $p < 0.05$. ** $p < 0.01$.

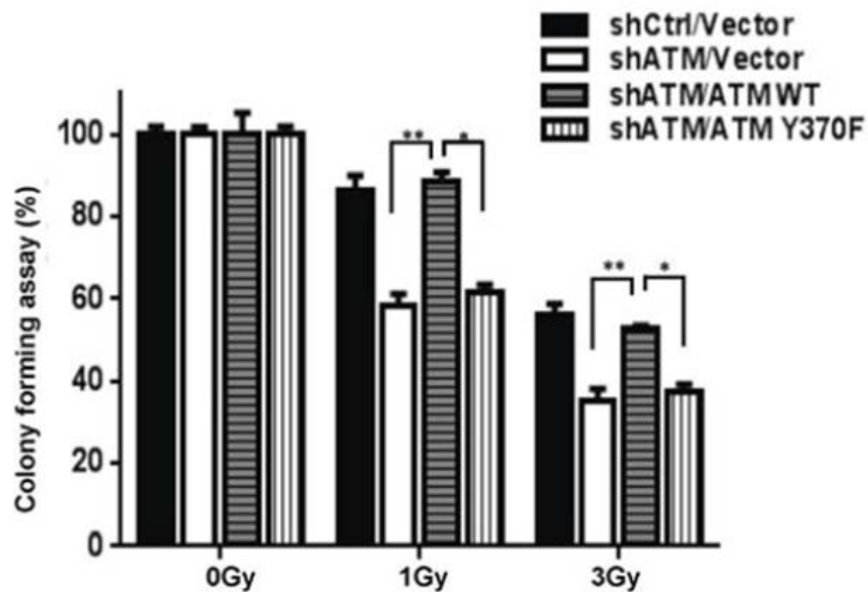


Figure 13 ATM Y370 phosphorylation regulates radio-sensitivity.

B. Western blot analysis of ATM-depleted HeLa cells with restoration of vector control, Flag-tagged ATM WT or Y370F.

Vector control, Flag-tagged ATM WT, or Y370F was restored in ATM-depleted HeLa cells (HeLa shATM). After transfection for forty-eight hours, cell lysates were harvested for Western blot analysis with indicated antibodies.

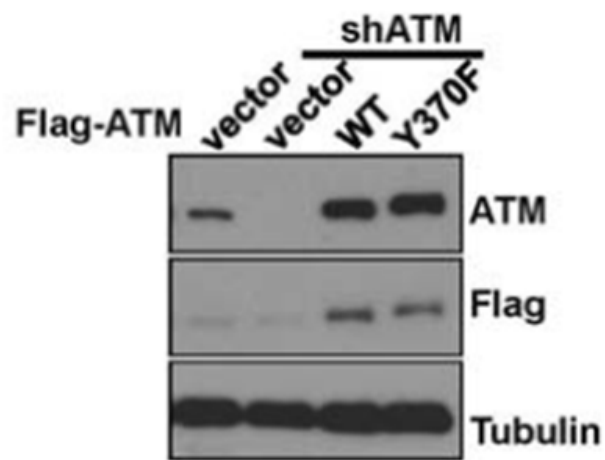
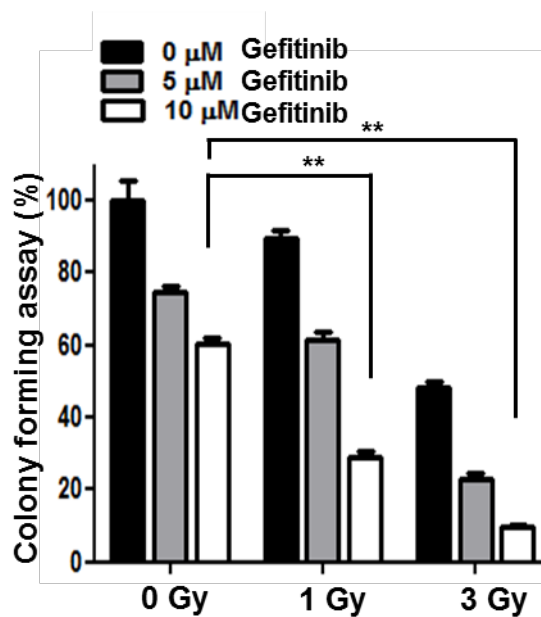


Figure 13 ATM Y370 phosphorylation regulates radio-sensitivity.

C. EGFR kinase activity is required for ATM-mediated radiosensitivity.

Colony-forming assay of HeLa cells treated with the indicated concentration of gefitinib with increasing doses of IR. The cell survival rate was presented as mean value \pm SD. $n = 3$. $**p < 0.01$.



CHAPTER 13 SUMMARY AND DISCUSSION

13.1 Summary

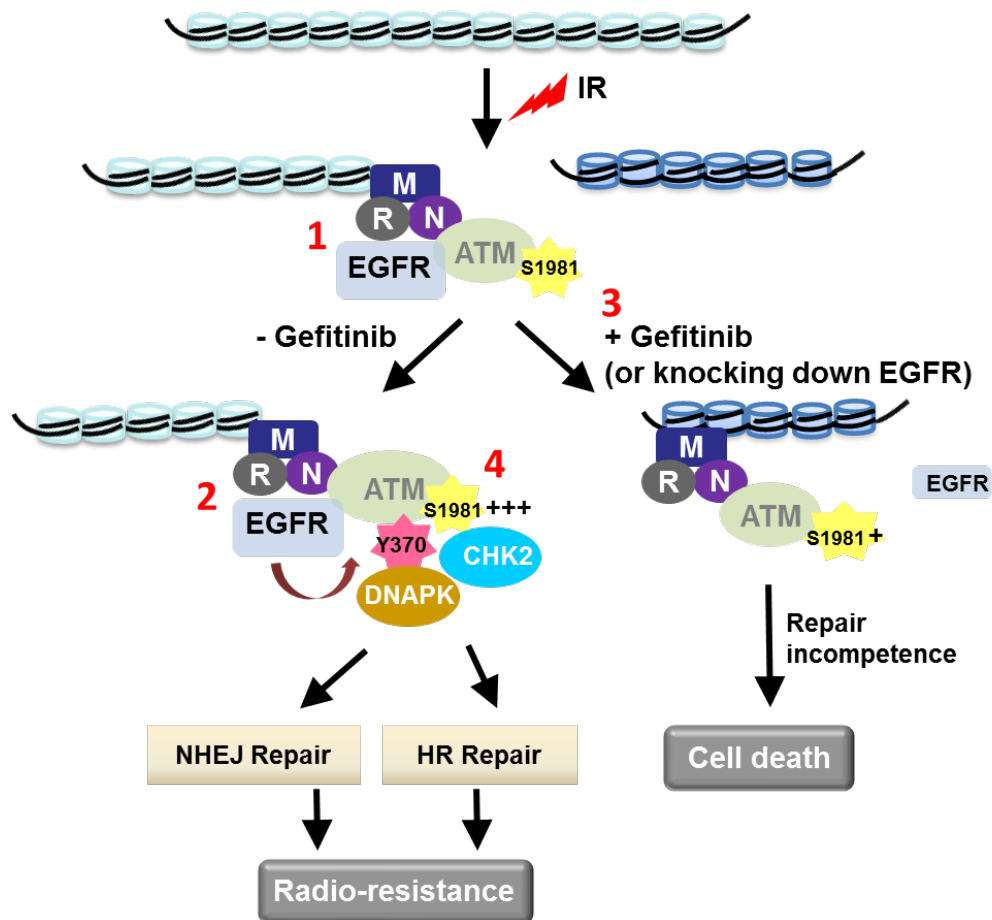
Ataxia telangiectasia-mutated (ATM) functions as one of the upstream serine/threonine kinases in DNA damage response. Upon formation of DNA double strand breaks (DSBs), ATM is rapidly activated and autophosphorylated at serine 1981 which then mediates downstream signaling pathway. However, how ATM is regulated remains unclear. As shown in the model below (Figure 14), we demonstrate that

1. ATM associates with and is phosphorylated at tyrosine 370 (Y370) by nuclear epidermal growth factor receptor (EGFR) upon IR stimulation.
2. ATM co-localizes with EGFR at DSBs by using multiple methods including chromatin immunoprecipitation, KillerRed system, and laser irradiation.
3. Knocking down EGFR or EGFR kinase inhibitor gefitinib treatment not only impairs ATM and downstream CHK2 and DNA-PKcs foci formation but also reduces DNA repair efficiency.
4. EGFR-mediated ATM Y370 phosphorylation facilitates ATM, DNA-PKcs and CHK2 foci formation, NBS1 phosphorylation, and enhances radiotherapy resistance.

Together, we unravel an EGFR mediated novel mechanism of ATM activation, downstream signal transduction, and DNA repair efficiency.

Figure 14 Propose model showing the role of EGFR in ATM-mediated DNA damage response.

EGFR-mediated ATM Y370 phosphorylation facilitates ATM S1981 autophosphorylation (indicated by “+”) and ATM-mediated DNA damage response such as DNA-PKcs and CHK2 IRIF. Pretreatment of EGFR kinase inhibitor (gefitinib) blocks ATM Y370 phosphorylation reduces ATM S1981 autophosphorylation and impairs ATM-mediated DNA damage response.



13.2 The role of EGFR in cancer cell radio-sensitivity.

The current report reveals a tyrosine phosphorylation of ATM at Y370 by EGFR after IR stimulation. The ATM Y370 phosphorylation event facilitates not only ATM activation but also ATM-mediated downstream DDR (Figure 14). Our data indicate that EGFR-mediated ATM Y370 phosphorylation confers radiotherapy resistance in cancer cells and suggest ATM phospho-Y370 could serve as a marker to stratify patients for rational combinational therapy of IR and TKI treatment.

Two different groups have previously reported that erlotinib or gefitinib pretreatment increases radio-sensitivities of triple-negative breast cancer (TNBC) and non-small cell lung cancer (NSCLC) cells, respectively (Lee et al., 2012; Park et al., 2010). The data presented in these studies substantiate the role of EGFR kinase activity in radiotherapy resistance by demonstrating that inactivation of EGFR kinase activity enhances radio-sensitivities of TNBC and NSCLC cells. In fact, Das and colleagues demonstrated EGFR tyrosine kinase domain mutants in NSCLC cells impaired radiation-induced EGFR nuclear translocation and significantly delayed DSB repair efficiency (Das et al., 2007), further providing evidence to support the role of nuclear EGFR in DDR in our study. Together, our results and other group's data all indicate that EGFR kinase activity plays an important role in mediating various types of cancer cell radio-sensitivity.

13.3 Tyrosine kinases mediate ATM signaling.

In 2013, Jackson's group demonstrated that c-Abl regulates ATM signaling through Y44 phosphorylation of the protein acetyltransferase KAT5 (also known as TIP60), which increases the acetylation level of ATM (Kaidi and Jackson, 2013). We showed that pretreatment of c-Abl kinase inhibitor imatinib did not reduce ATM tyrosine phosphorylation as opposed to EGFR kinase inhibitors gefitinib and AG1478 (Figure 5A), suggesting that c-Abl may not regulate ATM signaling by direct phosphorylation of ATM. Thus, tyrosine kinase such as EGFR can directly phosphorylate ATM or like c-Abl can indirectly regulate ATM through phosphorylation of KAT5.

13.4 The connections among ATM, DNA-PKcs and EGFR.

EGFR has been reported to associate with DNA-PKcs, one of the major serine/threonine kinases in DDR, to mediate non-homologous end joining after IR, but the detailed mechanism remains unclear (Bandyopadhyay et al., 1998; Dittmann et al., 2005a). Chen and colleagues also demonstrated that activated ATM is essential for DNA-PKcs activation (Chen et al., 2007). They found that endogenous ATM-depleted cells mediated by shRNA or A-T cells decreases DNA-PKcs threonine 2609 (T2609) phosphorylation, which is critical for its role in DSB repair and radiation resistance. Here, we identified a mechanism in DNA-PKcs IRIF in DDR through EGFR-mediated Y370

phosphorylation of ATM, filling the gap between ATM signaling and DNA-PKcs T2609 phosphorylation. Taken together, our findings indicate that EGFR plays a critical role in DDR and that phospho-ATM-Y370 has the potential to serve as a biomarker in radiotherapy or chemotherapy combined with targeted EGFR inhibitors in cancer treatments.

CHAPTER 14 FUTURE DIRECTIONS

14.1 Hit and stay model

Our results indicate EGFR associates and co-localizes with ATM at DSBs upon IR stimulation (Figures 6B, 6C and 8B). Interestingly, we found that ATM Y370F, a phosphorylation defective mutant of ATM, impairs association with Myc-tagged EGFR in co-IP experiment (Figure 11A) and pretreatment of EGFR kinase inhibitor gefitinib also reduces interaction between endogenous EGR and ATM (Figures 6B and 6C), suggesting both EGFR kinase activity and ATM Y370 phosphorylation are required for EGFR-ATM interaction. These findings raise our interest to figure out whether EGFR mediates ATM and other DDR proteins through a “Hit and Stay model”.

In this Hit and Stay model, we hypothesize that EGFR binds to and phosphorylates ATM at Y370 in response to DSBs induction. Instead of leaving the protein complex, EGFR keeps binding with the protein complex and further phosphorylates other DDR proteins located at DSBs. Indeed, we showed that EGFR not only associates with ATM but also MRN (Mre11-Rad50-NBS1) complex in the nucleus after IR treatment (Figure 8D). MRN complex serves as a critical component in DDR by accumulating at DSBs in a very short time after DSBs formation, tethering broken DNA ends, and

recruiting more DDR proteins like ATM or γ -H2AX to DSBs for subsequently signal transduction (Bensimon et al., 2011; Williams et al., 2010).

To current knowledge, three tyrosine phosphorylation sites (Y16, Y197 and Y429) of NBS1 and Y1125 phosphorylation of Rad50 were identified by mass spectrometry carried out by different groups (Chen et al., 2009; Li et al., 2009b; Olsen et al., 2010; Phanstiel et al., 2011; Rigbolt et al., 2011). However, tyrosine kinases responsible for those phosphorylation events or subsequent detailed functions in DDR are not clear. It requires further experiments to elucidate whether EGFR phosphorylates those DDR proteins and contributes their functions in DDR.

14.2 ATM conformational changes in response to DSBs formation.

ATM is known to be activated by inductions of DSBs formation, dissociated from inactive homodimer to active monomer and autophosphorylated at S367, S1893, and S1981 (Bakkenist and Kastan, 2003; Bensimon et al., 2011; Kozlov et al., 2011). However, how dimeric and inactive ATM dissociating as monomeric ATM with activity in response to chromosome structural changes remains unclear. In current report, we demonstrated that phosphorylation defective mutant ATM, Y370F, fails to associate with EGFR (Figure 11A) and impairs ATM and downstream CHK2 IRIIF (Figures 12A and 12C), implying EGFR-mediating ATM Y370

phosphorylation may participate into activation and dissociation of dimeric ATM.

To investigate whether ATM Y370 phosphorylation is required for dimeric ATM dissociation, we can purify recombinant Flag-tagged ATM WT and HA-tagged ATM Y370F proteins from 293T transfectants cells (Lee and Paull, 2004). After sequential purification, we can analyze those ATM protein compositions by using native gel electrophoresis followed by western blotting. Besides, those purified ATM proteins can be used in *in vitro* binding assay with MRN protein complex and the results may bring us hints regarding the connections in those DDR proteins.

14.3 Combination therapy of targeting nuclear EGFR and radiation

Nuclear EGFR had been linked to tumor progression, poor prognosis, radiotherapy resistance in types of cancer (Brand et al., 2013; Wang et al., 2010). Previous reports indicated that PKC ϵ -mediated EGFR Y654 phosphorylation, Src family kinase (SFK)-mediated EGFR Y1101 phosphorylation and AKT-mediated EGFR S229 phosphorylation all contribute to EGFR nuclear translocation in human NSCLC and breast cancer (Huang et al., 2011; Iida et al., 2013; Li et al., 2009a). Our findings indicate nuclear EGFR-mediated ATM Y370 phosphorylation is essential for radiotherapy resistance (Figures 13A and 13C). Therefore, we hypothesize

impeding EGFR nuclear translocation by using inhibitors targeting PKC ϵ , SFK and AKT may sensitize cancer cell to radiation treatment.

To this end, we can first test whether PKC ϵ inhibitors Enzastaurin, Midostaurin, SFK inhibitor Dasatinib and AKT inhibitor MK2206 block radiation-induced EGFR nuclear translocation and ATM Y370 phosphorylation. Next, we can further investigate whether combination of those inhibitors with radiation enhance cancer cell radiotherapy sensitivity *in vivo* and *in vitro*. Collectively, blockade of EGFR nuclear translocation combines with radiation may benefit radiotherapy or chemotherapy resistant cancer patients and improve their survival rate in the future.

BIBLIOGRAPHY

- Ahn, J.Y., Schwarz, J.K., Piwnica-Worms, H., and Canman, C.E. (2000). Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer research* 60, 5934-5936.
- Bakkenist, C.J., and Kastan, M.B. (2003). DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421, 499-506.
- Bandyopadhyay, D., Mandal, M., Adam, L., Mendelsohn, J., and Kumar, R. (1998). Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. *The Journal of biological chemistry* 273, 1568-1573.
- Barlow, C., Brown, K.D., Deng, C.X., Tagle, D.A., and Wynshaw-Boris, A. (1997). Atm selectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways. *Nature genetics* 17, 453-456.
- Bennetzen, M.V., Larsen, D.H., Bunkenborg, J., Bartek, J., Lukas, J., and Andersen, J.S. (2010). Site-specific phosphorylation dynamics of the nuclear proteome during the DNA damage response. *Molecular & cellular proteomics : MCP* 9, 1314-1323.
- Bensimon, A., Aebersold, R., and Shiloh, Y. (2011). Beyond ATM: the protein kinase landscape of the DNA damage response. *FEBS letters* 585, 1625-1639.
- Bensimon, A., Schmidt, A., Ziv, Y., Elkon, R., Wang, S.Y., Chen, D.J., Aebersold, R., and Shiloh, Y. (2010). ATM-dependent and -independent dynamics of the nuclear phosphoproteome after DNA damage. *Science signaling* 3, rs3.

Bernier, J., Bentzen, S.M., and Vermorken, J.B. (2009). Molecular therapy in head and neck oncology. *Nature reviews Clinical oncology* 6, 266-277.

Bogdan, S., and Klambt, C. (2001). Epidermal growth factor receptor signaling. *Current biology : CB* 11, R292-295.

Bonner, J.A., Harari, P.M., Giralt, J., Azarnia, N., Shin, D.M., Cohen, R.B., Jones, C.U., Sur, R., Raben, D., Jassem, J., Ove, R., Kies, M.S., Baselga, J., Youssoufian, H., Amellal, N., Rowinsky, E.K., and Ang, K.K. (2006). Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *The New England journal of medicine* 354, 567-578.

Brand, T.M., Iida, M., Luthar, N., Starr, M.M., Huppert, E.J., and Wheeler, D.L. (2013). Nuclear EGFR as a molecular target in cancer. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 108, 370-377.

Carpentier, P., Violot, S., Blanchoin, L., and Bourgeois, D. (2009). Structural basis for the phototoxicity of the fluorescent protein KillerRed. *FEBS Lett* 583, 2839-2842.

Chen, B.P., Uematsu, N., Kobayashi, J., Lerenthal, Y., Krempler, A., Yajima, H., Lobrich, M., Shiloh, Y., and Chen, D.J. (2007). Ataxia telangiectasia mutated (ATM) is essential for DNA-PKcs phosphorylations at the Thr-2609 cluster upon DNA double strand break. *The Journal of biological chemistry* 282, 6582-6587.

Chen, R.Q., Yang, Q.K., Lu, B.W., Yi, W., Cantin, G., Chen, Y.L., Fearn, C., Yates, J.R., 3rd, and Lee, J.D. (2009). CDC25B mediates rapamycin-induced oncogenic responses in cancer cells. *Cancer research* 69, 2663-2668.

Chinnaiyan, P., Huang, S., Vallabhaneni, G., Armstrong, E., Varambally, S., Tomlins, S.A., Chinnaiyan, A.M., and Harari, P.M. (2005). Mechanisms of enhanced radiation response following epidermal growth factor receptor signaling inhibition by erlotinib (Tarceva). *Cancer research* 65, 3328-3335.

Cimprich, K.A., and Cortez, D. (2008). ATR: an essential regulator of genome integrity. *Nature reviews Molecular cell biology* 9, 616-627.

Cortez, D., Wang, Y., Qin, J., and Elledge, S.J. (1999). Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science* 286, 1162-1166.

Daniel, J.A., Pellegrini, M., Lee, J.H., Paull, T.T., Feigenbaum, L., and Nussenzweig, A. (2008). Multiple autophosphorylation sites are dispensable for murine ATM activation in vivo. *The Journal of cell biology* 183, 777-783.

Das, A.K., Chen, B.P., Story, M.D., Sato, M., Minna, J.D., Chen, D.J., and Nirodi, C.S. (2007). Somatic mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) abrogate EGFR-mediated radioprotection in non-small cell lung carcinoma. *Cancer research* 67, 5267-5274.

Derheimer, F.A., and Kastan, M.B. (2010). Multiple roles of ATM in monitoring and maintaining DNA integrity. *FEBS letters* 584, 3675-3681.

Ditch, S., and Paull, T.T. (2012). The ATM protein kinase and cellular redox signaling: beyond the DNA damage response. *Trends in biochemical sciences* 37, 15-22.

Dittmann, K., Mayer, C., Fehrenbacher, B., Schaller, M., Raju, U., Milas, L., Chen, D.J., Kehlbach, R., and Rodemann, H.P. (2005a). Radiation-induced epidermal growth factor receptor nuclear import is linked to activation of DNA-dependent protein kinase. *The Journal of biological chemistry* 280, 31182-31189.

Dittmann, K., Mayer, C., Kehlbach, R., and Rodemann, H.P. (2008a). Radiation-induced caveolin-1 associated EGFR internalization is linked with nuclear EGFR transport and activation of DNA-PK. *Molecular cancer* 7, 69.

Dittmann, K., Mayer, C., and Rodemann, H.P. (2005b). Inhibition of radiation-induced EGFR nuclear import by C225 (Cetuximab) suppresses DNA-PK activity. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology* 76, 157-161.

Dittmann, K.H., Mayer, C., Ohneseit, P.A., Raju, U., Andratschke, N.H., Milas, L., and Rodemann, H.P. (2008b). Celecoxib induced tumor cell radiosensitization by inhibiting radiation induced nuclear EGFR transport and DNA-repair: a COX-2 independent mechanism. *International journal of radiation oncology, biology, physics* 70, 203-212.

Dobbs, T.A., Tainer, J.A., and Lees-Miller, S.P. (2010). A structural model for regulation of NHEJ by DNA-PKcs autophosphorylation. *DNA repair* 9, 1307-1314.

Ferguson, K.M., Berger, M.B., Mendrola, J.M., Cho, H.S., Leahy, D.J., and Lemmon, M.A. (2003). EGF activates its receptor by removing interactions that autoinhibit ectodomain dimerization. *Molecular cell* 11, 507-517.

Flynn, R.L., and Zou, L. (2011). ATR: a master conductor of cellular responses to DNA replication stress. *Trends in biochemical sciences* 36, 133-140.

Garrett, T.P., McKern, N.M., Lou, M., Elleman, T.C., Adams, T.E., Lovrecz, G.O., Zhu, H.J., Walker, F., Frenkel, M.J., Hoyne, P.A., Jorissen, R.N., Nice, E.C., Burgess, A.W., and Ward, C.W. (2002). Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell* 110, 763-773.

Guo, Z., Kozlov, S., Lavin, M.F., Person, M.D., and Paull, T.T. (2010). ATM activation by oxidative stress. *Science* 330, 517-521.

Hanada, N., Lo, H.W., Day, C.P., Pan, Y., Nakajima, Y., and Hung, M.C. (2006). Co-regulation of B-Myb expression by E2F1 and EGF receptor. *Molecular carcinogenesis* 45, 10-17.

Harari, P.M., and Huang, S.M. (2001). Head and neck cancer as a clinical model for molecular targeting of therapy: combining EGFR blockade with radiation. *International journal of radiation oncology, biology, physics* 49, 427-433.

Holbro, T., Civenni, G., and Hynes, N.E. (2003). The ErbB receptors and their role in cancer progression. *Experimental cell research* 284, 99-110.

Hsu, S.C., and Hung, M.C. (2007). Characterization of a novel tripartite nuclear localization sequence in the EGFR family. *The Journal of biological chemistry* 282, 10432-10440.

Huang, S.M., Bock, J.M., and Harari, P.M. (1999). Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. *Cancer research* 59, 1935-1940.

Huang, S.M., and Harari, P.M. (2000). Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clinical cancer research : an official journal of the American Association for Cancer Research* 6, 2166-2174.

Huang, T.H., Huo, L., Wang, Y.N., Xia, W., Wei, Y., Chang, S.S., Chang, W.C., Fang, Y.F., Chen, C.T., Lang, J.Y., Tu, C., Wang, Y., Hsu, M.C., Kuo, H.P., Ko, H.W., Shen, J., Lee, H.H., Lee, P.C., Wu, Y., Chen, C.H., and Hung, M.C. (2013). Epidermal growth factor receptor potentiates MCM7-mediated DNA replication through tyrosine phosphorylation of Lyn kinase in human cancers. *Cancer cell* 23, 796-810.

Huang, W.C., Chen, Y.J., Li, L.Y., Wei, Y.L., Hsu, S.C., Tsai, S.L., Chiu, P.C., Huang, W.P., Wang, Y.N., Chen, C.H., Chang, W.C., Chang, W.C., Chen, A.J., Tsai, C.H., and Hung, M.C. (2011). Nuclear translocation of epidermal growth factor receptor by Akt-dependent phosphorylation enhances breast cancer-resistant protein expression in gefitinib-resistant cells. *The Journal of biological chemistry* 286, 20558-20568.

Hung, L.Y., Tseng, J.T., Lee, Y.C., Xia, W., Wang, Y.N., Wu, M.L., Chuang, Y.H., Lai, C.H., and Chang, W.C. (2008). Nuclear epidermal growth factor receptor (EGFR) interacts with signal transducer and activator of transcription 5 (STAT5) in activating Aurora-A gene expression. *Nucleic acids research* 36, 4337-4351.

Iida, M., Brand, T.M., Campbell, D.A., Li, C., and Wheeler, D.L. (2013). Yes and Lyn play a role in nuclear translocation of the epidermal growth factor receptor. *Oncogene* 32, 759-767.

Iijima, K., Ohara, M., Seki, R., and Tauchi, H. (2008). Dancing on damaged chromatin: functions of ATM and the RAD50/MRE11/NBS1 complex in cellular responses to DNA damage. *Journal of radiation research* 49, 451-464.

Jackson, S.P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature* 461, 1071-1078.

Jorissen, R.N., Walker, F., Pouliot, N., Garrett, T.P., Ward, C.W., and Burgess, A.W. (2003). Epidermal growth factor receptor: mechanisms of activation and signalling. *Experimental cell research* 284, 31-53.

Kaidi, A., and Jackson, S.P. (2013). KAT5 tyrosine phosphorylation couples chromatin sensing to ATM signalling. *Nature* 498, 70-74.

Kang, K.B., Zhu, C., Wong, Y.L., Gao, Q., Ty, A., and Wong, M.C. (2012). Gefitinib radiosensitizes stem-like glioma cells: inhibition of epidermal growth factor receptor-Akt-DNA-PK signaling, accompanied by inhibition of DNA double-strand break repair. *International journal of radiation oncology, biology, physics* 83, e43-52.

Kastan, M.B., and Lim, D.S. (2000). The many substrates and functions of ATM. *Nature reviews Molecular cell biology* 1, 179-186.

Khanna, K.K., Keating, K.E., Kozlov, S., Scott, S., Gatei, M., Hobson, K., Taya, Y., Gabrielli, B., Chan, D., Lees-Miller, S.P., and Lavin, M.F. (1998). ATM associates with and phosphorylates p53: mapping the region of interaction. *Nature genetics* 20, 398-400.

Kim, H.P., Yoon, Y.K., Kim, J.W., Han, S.W., Hur, H.S., Park, J., Lee, J.H., Oh, D.Y., Im, S.A., Bang, Y.J., and Kim, T.Y. (2009). Lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor, downregulates thymidylate synthase by inhibiting the nuclear translocation of EGFR and HER2. *PloS one* 4, e5933.

Kitagawa, R., Bakkenist, C.J., McKinnon, P.J., and Kastan, M.B. (2004). Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-BRCA1 pathway. *Genes & development* 18, 1423-1438.

Kozlov, S.V., Graham, M.E., Jakob, B., Tobias, F., Kijas, A.W., Tanuji, M., Chen, P., Robinson, P.J., Taucher-Scholz, G., Suzuki, K., So, S., Chen, D., and Lavin, M.F. (2011). Autophosphorylation and ATM activation: additional sites add to the complexity. *The Journal of biological chemistry* 286, 9107-9119.

Kozlov, S.V., Graham, M.E., Peng, C., Chen, P., Robinson, P.J., and Lavin, M.F. (2006). Involvement of novel autophosphorylation sites in ATM activation. *The EMBO journal* 25, 3504-3514.

Lan, L., Nakajima, S., Komatsu, K., Nussenzweig, A., Shimamoto, A., Oshima, J., and Yasui, A. (2005). Accumulation of Werner protein at DNA double-strand breaks in human cells. *Journal of cell science* 118, 4153-4162.

Lan, L., Nakajima, S., Oohata, Y., Takao, M., Okano, S., Masutani, M., Wilson, S.H., and Yasui, A. (2004). In situ analysis of repair processes for oxidative DNA damage in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* 101, 13738-13743.

Lan, L., Nakajima, S., Wei, L., Sun, L., Hsieh, C.L., Sobol, R.W., Bruchez, M., Van Houten, B., Yasui, A., and Levine, A.S. (2013). Novel method for site-specific induction of oxidative DNA damage reveals differences in recruitment of repair proteins to heterochromatin and euchromatin. *Nucleic acids research*.

Lan, L., Ui, A., Nakajima, S., Hatakeyama, K., Hoshi, M., Watanabe, R., Janicki, S.M., Ogiwara, H., Kohno, T., Kanno, S., and Yasui, A. (2010). The ACF1 complex is required for DNA double-strand break repair in human cells. *Molecular cell* 40, 976-987.

Lavin, M.F., and Kozlov, S. (2007). ATM activation and DNA damage response. *Cell cycle* 6, 931-942.

Lee, J.H., and Paull, T.T. (2004). Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science* 304, 93-96.

Lee, J.H., and Paull, T.T. (2005). ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science* 308, 551-554.

- Lee, J.H., and Paull, T.T. (2007). Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. *Oncogene* 26, 7741-7748.
- Lee, M.J., Ye, A.S., Gardino, A.K., Heijink, A.M., Sorger, P.K., MacBeath, G., and Yaffe, M.B. (2012). Sequential application of anticancer drugs enhances cell death by rewiring apoptotic signaling networks. *Cell* 149, 780-794.
- Lempiainen, H., and Halazonetis, T.D. (2009). Emerging common themes in regulation of PIKKs and PI3Ks. *The EMBO journal* 28, 3067-3073.
- Li, C., Iida, M., Dunn, E.F., Ghia, A.J., and Wheeler, D.L. (2009a). Nuclear EGFR contributes to acquired resistance to cetuximab. *Oncogene* 28, 3801-3813.
- Li, H., Ren, Z., Kang, X., Zhang, L., Li, X., Wang, Y., Xue, T., Shen, Y., and Liu, Y. (2009b). Identification of tyrosine-phosphorylated proteins associated with metastasis and functional analysis of FER in human hepatocellular carcinoma cells. *BMC cancer* 9, 366.
- Lim, D.S., Kim, S.T., Xu, B., Maser, R.S., Lin, J., Petrini, J.H., and Kastan, M.B. (2000). ATM phosphorylates p95/nbs1 in an S-phase checkpoint pathway. *Nature* 404, 613-617.
- Lin, H.Q., Meriaty, H., and Katsifis, A. (2011). Prediction of synergistic antitumour effect of gefitinib and radiation in vitro. *Anticancer research* 31, 2883-2888.
- Lin, S.Y., Makino, K., Xia, W., Matin, A., Wen, Y., Kwong, K.Y., Bourguignon, L., and Hung, M.C. (2001). Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nature cell biology* 3, 802-808.

Linggi, B., and Carpenter, G. (2006). ErbB receptors: new insights on mechanisms and biology. *Trends in cell biology* 16, 649-656.

Liu, M., Lee, D.F., Chen, C.T., Yen, C.J., Li, L.Y., Lee, H.J., Chang, C.J., Chang, W.C., Hsu, J.M., Kuo, H.P., Xia, W., Wei, Y., Chiu, P.C., Chou, C.K., Du, Y., Dhar, D., Karin, M., Chen, C.H., and Hung, M.C. (2012). IKKalpha activation of NOTCH links tumorigenesis via FOXA2 suppression. *Molecular cell* 45, 171-184.

Lo, H.W., Ali-Seyed, M., Wu, Y., Bartholomeusz, G., Hsu, S.C., and Hung, M.C. (2006). Nuclear-cytoplasmic transport of EGFR involves receptor endocytosis, importin beta1 and CRM1. *Journal of cellular biochemistry* 98, 1570-1583.

Lo, H.W., Hsu, S.C., Ali-Seyed, M., Gunduz, M., Xia, W., Wei, Y., Bartholomeusz, G., Shih, J.Y., and Hung, M.C. (2005). Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer cell* 7, 575-589.

Loeffler-Ragg, J., Schwentner, I., Sprinzl, G.M., and Zwierzina, H. (2008). EGFR inhibition as a therapy for head and neck squamous cell carcinoma. *Expert opinion on investigational drugs* 17, 1517-1531.

Lopez-Contreras, A.J., and Fernandez-Capetillo, O. (2010). The ATR barrier to replication-born DNA damage. *DNA repair* 9, 1249-1255.

Lukas, J., Lukas, C., and Bartek, J. (2011). More than just a focus: The chromatin response to DNA damage and its role in genome integrity maintenance. *Nature cell biology* 13, 1161-1169.

MacPhail, S.H., Banath, J.P., Yu, T.Y., Chu, E.H., Lambur, H., and Olive, P.L. (2003). Expression of phosphorylated histone H2AX in cultured cell lines following exposure to X-rays. *International journal of radiation biology* 79, 351-358.

Mahaney, B.L., Meek, K., and Lees-Miller, S.P. (2009). Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. *The Biochemical journal* 417, 639-650.

Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., Shiloh, Y., Gygi, S.P., and Elledge, S.J. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. *Science* 316, 1160-1166.

Matsuoka, S., Rotman, G., Ogawa, A., Shiloh, Y., Tamai, K., and Elledge, S.J. (2000). Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proceedings of the National Academy of Sciences of the United States of America* 97, 10389-10394.

McGowan, C.H., and Russell, P. (2004). The DNA damage response: sensing and signaling. *Current opinion in cell biology* 16, 629-633.

Milas, L., Mason, K., Hunter, N., Petersen, S., Yamakawa, M., Ang, K., Mendelsohn, J., and Fan, Z. (2000). In vivo enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. *Clinical cancer research : an official journal of the American Association for Cancer Research* 6, 701-708.

Morrison, C., Sonoda, E., Takao, N., Shinohara, A., Yamamoto, K., and Takeda, S. (2000). The controlling role of ATM in homologous recombinational repair of DNA damage. *The EMBO journal* 19, 463-471.

Nagasawa, H., Little, J.B., Lin, Y.F., So, S., Kurimasa, A., Peng, Y., Brogan, J.R., Chen, D.J., Bedford, J.S., and Chen, B.P. (2011). Differential role of DNA-PKcs phosphorylations and kinase activity in radiosensitivity and chromosomal instability. *Radiation research* 175, 83-89.

Normanno, N., Bianco, C., Strizzi, L., Mancino, M., Maiello, M.R., De Luca, A., Caponigro, F., and Salomon, D.S. (2005). The ErbB receptors and their ligands in cancer: an overview. *Current drug targets* 6, 243-257.

Normanno, N., De Luca, A., Bianco, C., Strizzi, L., Mancino, M., Maiello, M.R., Carotenuto, A., De Feo, G., Caponigro, F., and Salomon, D.S. (2006). Epidermal growth factor receptor (EGFR) signaling in cancer. *Gene* 366, 2-16.

Offterdinger, M., Schofer, C., Weipoltshammer, K., and Grunt, T.W. (2002). c-erbB-3: a nuclear protein in mammary epithelial cells. *The Journal of cell biology* 157, 929-939.

Ogiso, H., Ishitani, R., Nureki, O., Fukai, S., Yamanaka, M., Kim, J.H., Saito, K., Sakamoto, A., Inoue, M., Shirouzu, M., and Yokoyama, S. (2002). Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell* 110, 775-787.

Olayioye, M.A., Neve, R.M., Lane, H.A., and Hynes, N.E. (2000). The ErbB signaling network: receptor heterodimerization in development and cancer. *The EMBO journal* 19, 3159-3167.

Olsen, J.V., Vermeulen, M., Santamaria, A., Kumar, C., Miller, M.L., Jensen, L.J., Gnad, F., Cox, J., Jensen, T.S., Nigg, E.A., Brunak, S., and Mann, M. (2010). Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Science signaling* 3, ra3.

Park, S.Y., Kim, Y.M., and Pyo, H. (2010). Gefitinib radiosensitizes non-small cell lung cancer cells through inhibition of ataxia telangiectasia mutated. *Molecular cancer* 9, 222.

Pellegrini, M., Celeste, A., Difilippantonio, S., Guo, R., Wang, W., Feigenbaum, L., and Nussenzweig, A. (2006). Autophosphorylation at serine 1987 is dispensable for murine Atm activation in vivo. *Nature* 443, 222-225.

Peng, G., Yim, E.K., Dai, H., Jackson, A.P., Burgt, I., Pan, M.R., Hu, R., Li, K., and Lin, S.Y. (2009). BRIT1/MCPH1 links chromatin remodelling to DNA damage response. *Nature cell biology* 11, 865-872.

Perry, J., and Kleckner, N. (2003). The ATRs, ATMs, and TORs are giant HEAT repeat proteins. *Cell* 112, 151-155.

Phanstiel, D.H., Brumbaugh, J., Wenger, C.D., Tian, S., Probasco, M.D., Bailey, D.J., Swaney, D.L., Tervo, M.A., Bolin, J.M., Ruotti, V., Stewart, R., Thomson, J.A., and Coon, J.J. (2011). Proteomic and phosphoproteomic comparison of human ES and iPS cells. *Nature methods* 8, 821-827.

Pletnev, S., Gurskaya, N.G., Pletneva, N.V., Lukyanov, K.A., Chudakov, D.M., Martynov, V.I., Popov, V.O., Kovalchuk, M.V., Wlodawer, A., Dauter, Z., and Pletnev, V. (2009). Structural basis for phototoxicity of the genetically encoded photosensitizer KillerRed. *J Biol Chem* 284, 32028-32039.

Raben, D., Helfrich, B., Chan, D.C., Ciardiello, F., Zhao, L., Franklin, W., Baron, A.E., Zeng, C., Johnson, T.K., and Bunn, P.A., Jr. (2005). The effects of cetuximab alone and in combination with radiation and/or chemotherapy in lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 11, 795-805.

Rigbolt, K.T., Prokhorova, T.A., Akimov, V., Henningsen, J., Johansen, P.T., Kratchmarova, I., Kassem, M., Mann, M., Olsen, J.V., and Blagoev, B. (2011). System-wide temporal characterization of the proteome and phosphoproteome of human embryonic stem cell differentiation. *Science signaling* 4, rs3.

Shiotani, B., and Zou, L. (2009). ATR signaling at a glance. *Journal of cell science* 122, 301-304.

Sun, Y., Xu, Y., Roy, K., and Price, B.D. (2007). DNA damage-induced acetylation of lysine 3016 of ATM activates ATM kinase activity. *Molecular and cellular biology* 27, 8502-8509.

Tanaka, T., Munshi, A., Brooks, C., Liu, J., Hobbs, M.L., and Meyn, R.E. (2008). Gefitinib radiosensitizes non-small cell lung cancer cells by suppressing cellular DNA repair capacity. *Clinical cancer research : an official journal of the American Association for Cancer Research* 14, 1266-1273.

Taneja, N., Davis, M., Choy, J.S., Beckett, M.A., Singh, R., Kron, S.J., and Weichselbaum, R.R. (2004). Histone H2AX phosphorylation as a predictor of radiosensitivity and target for radiotherapy. *The Journal of biological chemistry* 279, 2273-2280.

Tebbutt, N., Pedersen, M.W., and Johns, T.G. (2013). Targeting the ERBB family in cancer: couples therapy. *Nature reviews Cancer* 13, 663-673.

Tian, B., Yang, Q., and Mao, Z. (2009). Phosphorylation of ATM by Cdk5 mediates DNA damage signalling and regulates neuronal death. *Nature cell biology* 11, 211-218.

Uberall, I., Kolar, Z., Trojanec, R., Berkovcova, J., and Hajduch, M. (2008). The status and role of ErbB receptors in human cancer. *Experimental and molecular pathology* 84, 79-89.

Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y. (2003). Requirement of the MRN complex for ATM activation by DNA damage. *The EMBO journal* 22, 5612-5621.

Wang, S.C., and Hung, M.C. (2009). Nuclear translocation of the epidermal growth factor receptor family membrane tyrosine kinase receptors. *Clinical cancer research : an official journal of the American Association for Cancer Research* 15, 6484-6489.

Wang, S.C., Lien, H.C., Xia, W., Chen, I.F., Lo, H.W., Wang, Z., Ali-Seyed, M., Lee, D.F., Bartholomeusz, G., Ou-Yang, F., Giri, D.K., and Hung, M.C. (2004). Binding at and transactivation of the COX-2 promoter by nuclear tyrosine kinase receptor ErbB-2. *Cancer cell* 6, 251-261.

- Wang, S.C., Nakajima, Y., Yu, Y.L., Xia, W., Chen, C.T., Yang, C.C., McIntush, E.W., Li, L.Y., Hawke, D.H., Kobayashi, R., and Hung, M.C. (2006). Tyrosine phosphorylation controls PCNA function through protein stability. *Nature cell biology* 8, 1359-1368.
- Wang, Y.N., Yamaguchi, H., Hsu, J.M., and Hung, M.C. (2010). Nuclear trafficking of the epidermal growth factor receptor family membrane proteins. *Oncogene* 29, 3997-4006.
- Ward, I.M., Wu, X., and Chen, J. (2001). Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. *The Journal of biological chemistry* 276, 47755-47758.
- Williams, G.J., Lees-Miller, S.P., and Tainer, J.A. (2010). Mre11-Rad50-Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. *DNA repair* 9, 1299-1306.
- Xie, Y., and Hung, M.C. (1994). Nuclear localization of p185neu tyrosine kinase and its association with transcriptional transactivation. *Biochemical and biophysical research communications* 203, 1589-1598.
- Yaffe, M.B. (2002). Phosphotyrosine-binding domains in signal transduction. *Nature reviews Molecular cell biology* 3, 177-186.
- Yang, D.Q., and Kastan, M.B. (2000). Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1. *Nature cell biology* 2, 893-898.
- Yarden, Y., and Sliwkowski, M.X. (2001). Untangling the ErbB signalling network. *Nature reviews Molecular cell biology* 2, 127-137.

Yazdi, P.T., Wang, Y., Zhao, S., Patel, N., Lee, E.Y., and Qin, J. (2002). SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes & development* 16, 571-582.

Yu, Y.L., Chou, R.H., Wu, C.H., Wang, Y.N., Chang, W.J., Tseng, Y.J., Chang, W.C., Lai, C.C., Lee, H.J., Huo, L., Chen, C.H., and Hung, M.C. (2012). Nuclear EGFR suppresses ribonuclease activity of polynucleotide phosphorylase through DNAPK-mediated phosphorylation at serine 776. *The Journal of biological chemistry* 287, 31015-31026.

Zandi, R., Larsen, A.B., Andersen, P., Stockhausen, M.T., and Poulsen, H.S. (2007). Mechanisms for oncogenic activation of the epidermal growth factor receptor. *Cellular signalling* 19, 2013-2023.

Zhang, N., Chen, P., Khanna, K.K., Scott, S., Gatei, M., Kozlov, S., Watters, D., Spring, K., Yen, T., and Lavin, M.F. (1997). Isolation of full-length ATM cDNA and correction of the ataxia-telangiectasia cellular phenotype. *Proceedings of the National Academy of Sciences of the United States of America* 94, 8021-8026.

Zhang, X., Gureasko, J., Shen, K., Cole, P.A., and Kuriyan, J. (2006). An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 125, 1137-1149.

Zhou, B.B., Chaturvedi, P., Spring, K., Scott, S.P., Johanson, R.A., Mishra, R., Mattern, M.R., Winkler, J.D., and Khanna, K.K. (2000). Caffeine abolishes the mammalian G(2)/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. *The Journal of biological chemistry* 275, 10342-10348.

Zhou, B.B., and Elledge, S.J. (2000). The DNA damage response: putting checkpoints in perspective. *Nature* 408, 433-439.

Zou, Y., Peng, H., Zhou, B., Wen, Y., Wang, S.C., Tsai, E.M., and Hung, M.C. (2002). Systemic tumor suppression by the proapoptotic gene *bik*. *Cancer research* 62, 8-12.

VITA

Hong-Jen Lee was born in Taiwan on February 15, 1975. He received the degree of Bachelor of Science with a major in animal science from Chinese Culture University in 1997 and the degree of Master of Science with a major in biochemistry and molecular biology (Mentor: Jung-Yaw Lin Ph.D) from National Taiwan University in 2004. For the next three years, he worked as a senior research assistant in the Institute of Biotechnology and Pharmaceutical Research (Mentor: Xin Chen Ph.D) at National Health Research Institute in Taiwan. He received travel award from National Health Research Institute in 2007. In August of 2007, he entered The University of Texas Graduate School of Biomedical Sciences at Houston to pursue his doctoral degree in Dr. Mien-Chie Hung's lab in M. D. Anderson Cancer Center.